US005952474A

United States Patent [19]

Kayman et al.

[11] **Patent Number: 5,952,474**

[45] **Date of Patent: Sep. 14, 1999**

[54] **FUSION GLYCOPROTEINS**

[75] Inventors: **Samuel Kayman**, New York; **Abraham Pinter**, Brooklyn, both of N.Y.

[73] Assignee: **The Public Health Research Institute of the City of New York, Inc.**, New York, N.Y.

[21] Appl. No.: **08/886,642**

[22] Filed: **Jul. 1, 1997**

Related U.S. Application Data

[60] Division of application No. 08/110,300, Aug. 20, 1993, Pat. No. 5,643,756, which is a continuation-in-part of application No. 07/938,100, Aug. 28, 1992, abandoned.

[51] **Int. Cl.**[6] **C07K 14/16**; C07K 14/435; C12P 21/02; C12N 15/85

[52] **U.S. Cl.** **530/395**; 435/69.7; 435/320.1; 530/350

[58] **Field of Search** 530/350, 395; 435/69.7, 320.1, 325, 357; 536/23.4

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,701,416	10/1987	Nunberg	435/320.1
5,266,478	11/1993	Chang et al.	435/328
5,643,756	7/1997	Kayman et al.	435/69.7

FOREIGN PATENT DOCUMENTS

0 356 409 A2	2/1990	European Pat. Off. .
WO 93/14188	7/1993	WIPO .
WO 93/20221	10/1993	WIPO .
WO 94/05780	3/1994	WIPO .
WO 94/11524	5/1994	WIPO .
WO 94/27643	12/1994	WIPO .

OTHER PUBLICATIONS

Alberts et al., "Molecular Biology of The Cell," Garland Publishing, Inc., New York, p. 172 (1983).

Battini, J. et al., "Receptor Choice Determinants in the Envelope Glycoproteins of Amphotropic, Xenotropic, And Polytropic Murine Leukemia Viruses," Journal of Virology, vol. 66, No. 3, pp. 1468–1475 (1992).

Benjouad, A., et al., "Influence of Carbohydrate Moieties On The Immunogenicity of Human Immunodeficiency Virus Type I Recombinant gp160," Journal of Virology, vol. 66, No. 4, pp. 2473–2483 (1992).

Buchbinder, A., et al., "Synergy Between Human Monoclonal Antibodies To HIV Extends Their Effective Biologic Activity Against Homologous And Divergent Strains," AIDS Research And Human Retroviruses, vol. 8, No. 4, pp. 425–427 (1992).

Fenouillet, E., et al., "Role of N–Linked Glycans In The Interaction Between The Envelope Glycoprotein of Human Immunodeficiency Virus And Its CD4 Cellular Receptor." J. Exp. Med. vol. 169, pp. 807–822 (1989).

Fenouillet, E., et al., "Role of N–Linked Glycans of Envelope Glycoproteins In Infectivity of Human Immunodeficiency Virus Type 1," Journal of Virology, vol. 64, No. 6, pp. 2841–2848 (1990).

Fung, M.S.C., et al., "Identification And Characterization of A Neutralization Site Within The Second Variable Region of Human Immunodeficiency Virus Tuype 1 gp120," Journal of Virology, vol. 66, No. 2, pp. 848–856 (1992).

Haigwood, N.L., et al., "Native But Not Denatured Recombinant Human Immunodeficiency Virus Type 1 gp120 Generates Broad–Spectrum Neutralizing Antobodies In Baboons," Journal of Virology, vol. 66, No. 1, pp. 172–182 (1992).

Heard, J.M., et al., "An Amino–Terminal Fragment of The Friend Murine Leukemia Virus Envelope Glycoprotein Binds The Ecotropic Receptor," Journal of Virology, vol. 65, No. 8, pp. 4026–4032 (1992).

Ho, D.D., et al., "Another Discontinuous Epitode On Glycoprotein gp120 That Is Important In Human Immunodeficiency Virus Type 1 Neutralization Is Identified By A Monoclonal Antibody," Proc. Natl. Acad. Sci., U.S.A., vol. 88, pp. 8949–8952 (1991).

Ho, D.D., et al., "Human Immunodeficiency Virus Neutralizing Antibodies Recognize Several Conserved Domains On The Envelope Glycoproteins," Journal of Virology, vol. 61, pp. 2024–2028 (1987).

Kayman, S.C., et al., "Mutational Analysis of N–Linked Glycosylation Sites of Friend Murine Leukemia Virus Envelope Protein," Journal of Virology, vol. 65, No. 10, pp. 5323–5232 (1991).

Kennedy, M.S., et al., "Analysis of Synergism/Antagonism Between HIV–1 Antibidy–Positive Human Sera And Soluble CD4 In Blocking HIV–1 Binding And Infectivity," AIDS Research And Human Retroviruses, vol. 7, No. 12, pp. 975–981 (1991).

Leonard, C.K., et al., "Assignment of Intrachain Disulfide Bonds And Characterization of Potential Glycosylation Sites of The Type 1 Recombinant Human Immunodeficiency Virus Envelope Glycoprotein (gp120) Expressed In Chinese Hamster Ovary Cells," Journal of Biological Chemistry, vol. 265, No. 18, pp. 10373–10382 (1990).

Lynch, C.M., et al., "Production of High–Titer Helper Virus–Free Retroviral Vectors By Cocultivation of Packaging Cells With Different Host Ranges," Journal of Virology, vol. 65, No. 7, pp. 3887–3890 (1991).

Mace, et al., "Retroviral Envelope Protein Fusions To Secreted And Menbrane markers," Virology, vol. 188, pp. 869–874 (1992).

Matthews, T.J., et al., "Interaction Between The Human T–Cell Lymphotropic Virus III^B Envelope Glycoprotein gp120 And The Surface Antigen CD4: Role of Carbohydrate In Binding And Cell Fusion," Proc. Natl. Acad. Sci., U.S.A., vol. 84, pp. 5424–5428 (1987).

(List continued on next page.)

Primary Examiner—Nancy Degen
Attorney, Agent, or Firm—Fish & Richardson P.C.

[57] **ABSTRACT**

Novel expression vectors are provided for expressing a fusion glycoprotein. The fusion glycoprotein contains the N-terminal globular domain of a retroviral env surface protein linked to a selected glycopeptide. Truncation glycoproteins as well as insertion glycoproteins are expressed using the vectors.

19 Claims, 19 Drawing Sheets

OTHER PUBLICATIONS

Ott, D., et al., "Basis For Receptor Specificity of Nonecotropic Murine Leukemia Virus Surface Gluycoprotein gp70^{su}," Journal of Virology, vol. 66, No. 8, pp. 4632–4638 (1992).

Pollard, S.R., et al., "Truncated Varaint of gp120 Bind CD4 With High Affinity And Suggest A Minimum CD4 Binding Region," The EMBO Journal, vol. 11, No. 2, pp. 585–591 (1992).

Posner, M., et al., "Development of An IgG–1 Human Monoclonal Antibody That Neutralizes HIV–1 Infectivity And Binding And Reacts With A Cell Surface Antigen Expressed By HIV–1 Infected Cells," VI Int'l. Conf. on AIDS, San Francisco, CA, Abstract Th.A.77 (1990).

Russell, S.J., et al., "Retroviral Vectors Displaying Functional Antibody Fragments," Nucleic Acids Research, vol. 21, No. 5, pp. 1081–1085 (1993).

Stephens, D.M., et al., "The Second Variable region of HIV–1 External Envelope Glycoprotein Contains A Neutralizing Epitope," VII Int'l Conf. on AIDS, Florence, Italy, Abstract TH.A.66 (1991).

Tilley, S.A., et al., "Human Monoclonal Antibodies Against The Putative CD4 Binding Site And The V3 Loop of HIV gp120 Act In Concert To Neutralize Virus," VIII Int'l Conf. On Aids, Florence, Italy, Abstract MA.70 (1991).

Tilley, S.A., et al., "Very Broadly Neutralizing Human Monoclonal Antibody (HuMAb) Against The CD4–Binding Site Of HIV–1 gp120," VIII Int'l. Conf. On AIDS/III STD World Congress, Amsterdam, The Netherlands, Abstract.

Tilley, S.A., et al., "Synergistic Neutralization of HIV–1 By Human Monoclonal Antibodies Against The V3 Loop And The CD4–Binding Site of gp120," AIDS Research And Human Retroviruses, vol. 8, No. 4, pp. 461–467 (1992).

Tilley, S.A., et al., "Potent Neutralization of HIV–1 By Human And Chimpanzee Monoclonal Antibodies Directed Against Three Distinct Epitope Clusters of gp120," Sixieme Coloque Des Cent Gardes, pp. 211–216 (1991).

Young, J.A.T., et al., "Efficient Incorporation of Human CD4 Protein Into Avian Leukosis Virus Particles," Science, vol. 250, pp. 1421–1423 (1990).

Wills, J.W., "Retro–Secretion of Recombinant Proteins," Nature, vol. 340, pp. 323–324 (1989).

Wu et al.; J. Virol. Apr. 1995; v. 69; p.. 2271–2278; Characterization of Neutralization Epitopes in the V2 Region of Human Immunodeficiency Virus Type 1, grp 120: Role of Glycosylation in the Correct Folding of the V1/V2 Domain.

Albert et al.; Molecular Biology of the Cell (Garland Publishing, Inc., New York, 1983) p. 172.

FIG. 1A

```
          |    10       |    20       |    30       |    40
     1    GCGCCAGTCC TCCGATAGAC TGAGTCGCCC GGGTACCCGT
    41    GTATCCAATA AATCCTCTTG CTGTTGCATC CGACTCGTGG
    81    TCTCGCTGTT CCTTGGGAGG GTCTCCTCAG AGTGATTGAC
   121    TACCCGTCTC GGGGGTCTTT CATTTGGGGG CTCGTCCGGG
   161    ATCTGGAGAC CCCTGCCCAG GGACCACCGA CCCACCACCG
   201    GGAGGTAAGC TGGCCAGCAA TTGTTCTGTG TCTGTCCATT
   241    GTCCTGTGTC TTTGATTGAT TTTATGCGCC TGTGTCTGTA
   281    CTAGTTGGCC GACTAGATTG GTATCTGGCG GATCCGTGGT
   321    GGAACTGACG AGTTCGAGAC ACCCGGCCGC AACCCTGGGA
   361    GACGTCCCAG GGACTTCGGG GGCCATTTTT GTGGCCCGGC
   401    CAGAGTCCAA CCATCCCGAT CGTTTTGGAC TCTTTGGTGC
   441    ACCCCCCTTA GAGGAGGGGT ATGTGGTTCT GGTAGGAGAC
   481    AGAGGGCTAA AACGGTTTCC GCCCCCGTCT GAGTTTTTGC
   521    TTTCGGTTTG GAACCGAAGC CGCGCCGCGC GTCTTGTCTG
   561    CTGCAGCATC GTTCTGTGTT GTCTCTGTTT GACTGTTTTT
   601    CTGTATTTGT CTGAAAACAT GGGCCAGGCT GTTACCACCC
   641    CCTTAAGTTT GACTTTAGAC CACTGGAAGG ATGTCGAACG
   681    GACAGCCCAC AACCTGTCGG TAGAGGTTAG AAAAAGGCGC
   721    TGGGTTACAT TCTGCTCTGC AGAATGGCCA ACCTTCAACG
   761    TCGGATGGCC ACGAGACGGC ACTTTTAACC CAGACATTAT
   801    TACACAGGTT AAGATCAAGG TCTTCTCACC TGGCCCACAT
   841    GGACATCCGG ATCAGGTCCC CTACATCGTG ACCTGGGAAG
   881    CTATAGCAGT AGACCCCCCT CCCTGGGTCA GACCCTTCGT
   921    GCACCCTAAA CCTCCCCTCT CTCTTCCCCC TTCAGCCCCC
   961    TCTCTCCCAC CTGAACCCCC ACTCTCGACC CCGCCCCAGT
  1001    CCTCCCTCTA TCCGGCTCTC ACTTCTCCTT TAAACACCAA
  1041    ACCTAGGCCT CAAGTCCTTC CTGATAGCGG AGGACCACTC
  1081    ATTGATCTAC TCACGGAGGA CCCTCCGCCT TACCGGGACC
  1121    CAGGGCCACC CTCTCCTGAC GGGAACGGCG ATAGCGGAGA
  1161    AGTGGCCCCT ACAGAAGGAG CCCCTGACCC TTCCCCAATG
  1201    GTATCCCGCC TGCGGGGAAG AAAAGAACCC CCCGTGGCGG
  1241    ATTCTACTAC CTCTCAGGCG TTCCCCCTTC GCCTGGGAGG
  1281    GAATGGACAG TATCAATACT GGCCATTTTC CTCCTCTGAC
  1321    CTCTATAACT GGAAAAATAA CAACCCCTCT TTCTCCGAGG
  1361    ACCCAGCTAA ATTGACAGCT TTGATCGAGT CCGTTCTCCT
  1401    TACTCATCAG CCCACTTGGG ATGACTGCCA ACAGCTATTA
  1441    GGGACCCTGC TGACGGGAGA AGAAAAACAG CGAGTGCTCC
  1481    TAGAGGCCCG AAAGGCGGTT CGAGGGGAGG ACGGACGCCC
  1521    AACTCAGCTG CCCAATGACA TTAATGATGC TTTTCCCTTG
  1561    GAACGTCCCG ACTGGGACTA CAACACCCAA CGAGGTAGGA
  1601    ACCACCTAGT CCACTATCGC CAGTTGCTCC TAGCGGGTCT
```

FIG. 1B

```
          |    10      |    20      |    30      |    40
1641   CCAAAACGCG GGCAGAAGCC CCACCAATTT GGCCAAGGTA
1681   AAAGGGATAA CCCAGGGACC TAATGAGTCT CCCTCAGCCT
1721   TTTTAGAGAG ACTCAAGGAG GCCTATCGCA GATACACTCC
1761   TTATGACCCT GAGGACCCAG GGCAAGAAAC CAATGTGGCC
1801   ATGTCATTCA TCTGGCAGTC CGCCCCGGAT ATCGGGCGAA
1841   AGTTAGAGCG GTTAGAAGAT TTGAAGAGTA AGACCTTAGG
1881   AGACTTAGTG AGGGAAGCTG AAAAGATCTT TAATAAACGA
1921   GAAACCCCGG AAGAAAGAGA GGAACGTATT AGGAGAGAAA
1961   CAGAGGAAAA GGAAGAACGC CGTAGGGCAG AGGATGTGCA
2001   GAGAGAGAAG GAGAGGGACC GCAGAAGACA TAGAGAAATG
2041   AGTAAGTTGC TGGCTACTGT CGTTAGCGGG CAGAGACAGG
2081   ATAGACAGGG AGGAGAGCGA AGGAGGCCCC AACTCGACCA
2121   CGACCAGTGT GCCTACTGCA AAGAAAAGGG ACATTGGGCT
2161   AGAGATTGCC CCAAGAAGCC AAGAGGACCC CGGGGACCAC
2201   GACCCCAGGC CTCCCTCCTG ACCTTAGACG ATTAGGGAGG
2241   TCAGGGTCAG GAGCCCCCCC CTGAACCCAG GATAACCCTC
2281   AGAGTCGGGG GGCAACCCGT CACCTTCCTA GTGGATACTG
2321   GGGCCCAACA CTCCGTGCTG ACCCAAAATC CTGGACCCCT
2361   AAGTGACAAG TCTGCCTGGG TCCAAGGGGC TACTGGAGGG
2401   AAGCGGTATC GCTGGACCAC GGATCGCCGA GTGCACCTAG
2441   CCACCGGTAA GGTCACCCAT TCTTTCCTCC ATGTACCAGA
2481   TTGCCCCTAT CCTCTGCTAG GAAGAGATTT GCTGACTAAA
2521   CTAAAAGCCC AAATTCACTT TGAGGGATCA GGAGCTCAGG
2561   TTGTGGGACC AATGGGACAG CCCCTGCAAG TGCTGACCCT
2601   AAACATAGAA GATGAGTATC GGCTACATGA GACCTCAAAA
2641   GGGCCAGATG TGCCTCTAGG GTCCACATGG CTCTCTGATT
2681   TTCCCCAGGC CTGGGCAGAA ACCGGGGGCA TGGGGCTGGC
2721   CGTTCGCCAA GCTCCTCTGA TCATACCTCT GAAGGCAACC
2761   TCTACCCCCG TGTCCATAAA ACAATACCCC ATGTCACAAG
2801   AAGCCAGACT GGGGATCAAG CCCCACATAC AGAGACTGCT
2841   GGATCAGGGA ATTCTGGTAC CCTGCCAGTC CCCCTGGAAC
2881   ACGCCCCTGC TACCCGTTAA GAAACCGGGG ACTAATGATT
2921   ATAGGCCTGT CCAGGATCTG AGAGAAGTCA ACAAGCGGGT
2961   GGAAGACATC CACCCCACCG TGCCCAACCC TTACAACCTC
3001   TTGAGCGGGC TCCCACCGTC CCACCAGTGG TACACTGTGC
3041   TTGACTTAAA AGATGCTTTT TTCTGCCTGA GACTCCACCC
3081   CACCAGTCAG TCTCTCTTCG CCTTTGAGTG GAGAGATCCA
3121   GAGATGGGAA TCTCAGGACA ATTAACCTGG ACCAGACTCC
3161   CGCAGGGTTT CAAAAACAGT CCCACCCTGT TTGATGAAGC
3201   CCTGCACAGG GACCTCGCAG ACTTCCGGAT CCAGCACCCA
3241   GACCTGATTC TGCTCCAGTA TGTAGATGAC TTACTGCTGG
3281   CCGCCACTTC TGAGCTTGAC TGTCAACAAG GTACGCGGGC
3321   CCTGTTACAA ACCCTAGGGG ACCTCGGATA TCGGGCCTCG
3361   GCCAAGAAAG CCCAAATTTG CCAGAAACAG GTCAAGTATC
```

FIG. 1C

```
          |    10      |    20      |    30      |    40
3401   TGGGGTATCT TCTAAAAGAG GGTCAGAGAT GGCTGACTGA
3441   GGCCAGAAAA GAGACTGTGA TGGGGCAGCC TACTCCGAAG
3481   ACCCCTCGAC AACTAAGGGA GTTCCTAGGG ACGGCAGGCT
3521   TCTGTCGCCT CTGGATCCCT GGGTTTGCAG AAATGGCAGC
3561   CCCCTTGTAC CCTCTCACCA AAACGGGGAC TCTGTTTGAG
3601   TGGGGCCCAG ACCAGCAAAA GGCCTACCAA GAGATCAAGC
3641   AGGCTCTCTT AACTGCCCCT GCCCTGGGAT TGCCAGACTT
3681   GACTAAGCCC TTCGAACTTT TTGTTGACGA GAAGCAGGGC
3721   TACGCCAAAG GTGTCCTAAC GCAAAAACTG GGGCCTTGGC
3761   GTCGGCCGGT GGCCTACCTG TCCAAAAAGC TAGACCCAGT
3801   GGCAGCTGGG TGGCCCCCTT GCCTACGGAT GGTAGCAGCC
3841   ATCGCCGTTC TGACCAAAGA CGCTGGCAAG CTCACCATGG
3881   GACAGCCACT AGTCATTCTG GCCCCCCATG CAGTAGAGGC
3921   ACTAGTTAAG CAACCCCCTG ATCGCTGGCT CTCCAACGCC
3961   CGAATGACCC ACTACCAGGC TCTGCTTCTG GACACGGACC
4001   GAGTCCAGTT CGGACCAATA GTGGCCCTAA ACCCAGCTAC
4041   GCTGCTCCCT CTACCTGAGG AGGGGCTGCA ACATGACTGC
4081   CTTGACATCT TGGCTGAAGC CCACGGAACT AGACCAGATC
4121   TTACGGACCA GCCTCTCCCA GACGCTGACC ACACCTGGTA
4161   CACAGATGGG AGCAGCTTCC TGCAAGAGGG GCAGCGCAAG
4201   GCCGGAGCAG CAGTAACCAC CGAGACCGAG GTAGTCTGGG
4241   CCAAAGCACT GCCAGCCGGG ACATCGGCCC AAAGAGCTGA
4281   GTTGATAGCG CTCACCCAAG CCTTAAAAAT GGCAGAAGGT
4321   AAGAAGCTGA ATGTTTACAC CGATAGCCGT TATGCTTTTG
4361   CCACTGCCCA TATTCACGGA GAAATATATA GAAGGCGCGG
4401   GTTGCTCACA TCAGAAGGAA AAGAAATCAA AAATAAGGAC
4441   GAGATCTTGG CCCTACTGAA GGCTCTCTTC CTGCCCAAAA
4481   GACTTAGCAT AATTCATTGC CCGGGACATC AGAAGGGAAA
4521   CCGCGCGGAG GCAAGGGGCA ACAGGATGGC CGACCAAGCG
4561   GCCCGAGAAG TAGCCACTAG AGAAACTCCA GAGACTTCCA
4601   CACTTCTGAT AGAAAATTCA GCCCCCTATA CTCATGAACA
4641   TTTTCACTAT ACGGTGACTG ACATAAAAGA TCTGACTAAA
4681   CTAGGGGCCA CTTATGACGA TGCAAAGAAG TGTTGGGTTT
4721   ATCAGGGAAA GCCTGTAATG CCTGATCAAT TCACCTTTGA
4761   ACTATTAGAT TTTCTTCATC AATTGACCCA CCTCAGTTTC
4800   TCAAAAACAA AGGCTCTTCT AGAAAGGAAC TACTGTCCTT
4841   ATTACATGCT GAACCGGGAT CGAACGCTCA AAGACATCAC
4881   TGAGACTTGC CAAGCCTGTG CACAGGTCAA TGCCAGCAAG
4921   TCTGCCGTCA AACAAGGGAC TAGAGTTCGA GGGCACCGAC
4961   CCGGCACCCA CTGGGAAATT GATTTCACTG AGGTAAAACC
5001   TGGCCTGTAT GGGTATAAAT ATCTTTTAGT TTTCATAGAC
5041   ACTTTCTCTG GATGGGTAGA AGCTTTCCCA ACCAAGAAAG
5081   AAACTGCCAA AGTTGTAACC AAGAAGCTAC TAGAAGAAAT
5121   CTTCCCCAGA TTCGGCATGC CACAGGTATT GGGAACCGAC
```

FIG. 1D

```
          |    10      |    20      |    30      |    40
5161   AATGGGCCTG CCTTCGTCTC CAAGGTAAGT CAGACAGTAG
5201   CCGATTTACT GGGGGTTGAT TGGAAACTAC ATTGTGCTTA
5241   CAGACCCCAG AGTTCAGGTC AGGTAGAAAG AATGAATAGG
5281   ACAATCAAGG AGACTTTAAC TAAATTGACG CTTGCAACTG
5321   GCTCTAGGGA CTGGGTGCTC CTGCTTCCCC TAGCCCTGTA
5361   TCGAGCCCGC AACACGCCGG GCCCCCATGG TCTCACCCCA
5401   TATGAAATCT TATATGGGGC ACCCCCGCCC CTTGTAAACT
5441   TCCCTGATCC TGACATGGCA AAGGTTACTC ATAACCCCTC
5481   TCTCCAAGCC CATTTACAGG CACTCTACCT GGTCCAGCAC
5521   GAAGTCTGGA GACCGTTGGC GGCAGCTTAC CAAGAACAAC
5561   TGGACCGGCC GGTAGTGCCT CACCCTTTCC GAGTCGGTGA
5601   CACAGTGTGG GTCCGCAGAC ACCAAACTAA AAATCTAGAA
5641   CCCCGCTGGA AAGGACCTTA TACCGTCCTA CTGACTACCC
5681   CCACCGCTCT CAAAGTGGAC GGCATTGCAG CGTGGATCCA
5721   CGCTGCCCAC GTAAAGGCTG CCGACACCAG GATTGAGCCA
5761   CCATCGGAAT CGACATGGCG TGTTCAACGC TCTCAAAATC
5801   CCCTAAAGAT AAGATTGACC CGCGGGACCT CCTAATCCCC
5841   TTAATTCTCT TCCTGTCTCT CAAAGGGGCC AGATCCGCAG
5881   CACCCGGCTC CAGCCCTCAC CAGGTCTACA ACATTACCTG
5921   GGAAGTGACC AATGGGGATC GGGAGACAGT ATGGGCAATA
5961   TCAGGCAACC ACCCTCTGTG GACTTGGTGG CCAGTCCTCA
6001   CCCCAGATTT GTGTATGTTA GCTCTCAGTG GGCCGCCCCA
6041   CTGGGGGCTA GAGTATCAGG CCCCCTATTC CTCGCCCCCG
6081   GGGCCCCCTT GTTGCTCAGG GAGCAGCGGG AACGTTGCAG
6121   GCTGTGCCAG AGACTGCAAC GAGCCCTTGA CCTCCCTCAC
6161   CCCTCGGTGC AACACTGCCT GGAACAGACT TAAGCTGGAC
6201   CAGGTAACTC ATAAATCAAG TGAGGGATTT TATGTCTGCC
6241   CCGGGTCACA TCGCCCCCGG GAAGCCAAGT CCTGTGGGGG
6281   TCCAGACTCC TTCTACTGTG CCTCTTGGGG CTGCGAGACA
6321   ACCGGTAGAG TATACTGGAA GCCCTCCTCT TCTTGGGACT
6361   ACATCACAGT AGACAACAAT CTCACCTCTA ACCAGGCTGT
6401   TCAGGTATGC AAAGACAATA AGTGGTGCAA TCCCTTGGCT
6441   ATCCGGTTTA CAAACGCCGG GAAACAGGTC ACCTCATGGA
6481   CAACTGGACA CTATTGGGGT CTACGTCTTT ATGTCTCTGG
6521   ACAGGACCCA GGGCTTACTT TCGGGATCCG ACTCAGTTAT
6561   CAAAATCTAG GACCTCGGAT CCCAATAGGA CCAAACCCCG
6601   TCCTGGCAGA CCAACTTTCG TTCCCGCTAC CTAATCCCCT
6641   ACCCAAACCT GCCAAGTCTC CCCCCGCCTC TAGTTCGACT
6681   CCCACATTGA TTTCCCCGTC CCCCACTCCC ACTCAGCCCC
6721   CGCCAGCAGG AACGGGAGAC AGATTACTAA ATCTAGTACA
6761   GGGAGCTTAC CAGGCACTCA ACCTTACCAA CCCTGATAAA
6801   ACTCAAGAGT GCTGGTTATG CCTAGTGTCT GGACCCCCCT
6841   ATTACGAGGG GGTTGCCGTC CTAGGTACTT ATTCCAACCA
6881   TACCTCTGCC CCAGCTAACT GCTCCGTGGC CTCCCAACAC
```

FIG. 1E

```
                |    10       |    20       |    30       |    40
6921    AAGCTGACCC TGTCCGAAGT GACTGGACGG GGACTCTGCA
6961    TAGGAACAGT CCCAAAAACT CACCAGGCCC TGTGCAACAC
7001    TACCCTTAAG GCAGGCAAAG GGTCTTACTA TCTAGTTGCC
7041    CCCACAGGAA CTATGTGGGC ATGTAACACT GGACTCACTC
7081    CATGCCTATC TGCCACCGTG CTTAATCGCA CCACTGACTA
7121    TTGCGTTCTC GTGGAATTAT GGCCCAGGGT CACCTACCAT
7161    CCTCCCAGTT ACGTCTATAG CCAGTTTGAA AAATCCCATA
7201    GACATAAAAG AGAACCAGTG TCCTTAACCT TGGCCTTATT
7241    ATTAGGTGGG CTAACTATGG GTGGCATCGC CGCGGGAGTA
7281    GGGACAGGAA CTACCGCCCT GGTCGCCACC CAGCAGTTTC
7321    AGCAGCTCCA TGCTGCCGTA CAAGATGATC TCAAAGAAGT
7361    CGAAAAGTCA ATTACTAACC TAGAAAAGTC TCTTACTTCG
7401    TTGTCTGAGG TTGTACTGCA GAATCGACGA GGCCTAGACC
7441    TGTTGTTCCT AAAAGAGGGA GGACTGTGTG CTGCCCTAAA
7481    AGAAGAATGT TGTTTCTATG CTGACCATAC AGGCCTAGTA
7521    AGAGATAGTA TGGCCAAATT AAGAGAGAGA CTCTCTCAGA
7561    GACAAAAACT ATTTGAGTCG AGCCAAGGAT GGTTCGAAGG
7601    ATGGTTTAAC AGATCCCCCT GGTTTACCAC GTTGATATCC
7641    ACCATCATGG GGCCTCTCAT TATACTCCTA CTAATTCTGC
7681    TTTTTGGACC CTGCATTCTT AATCGATTAG TTCAATTTGT
7721    TAAAGACAGG ATCTCAGTAG TCCAGGCTTT AGTCCTGACT
7761    CAACAATACC ACCAGCTAAA ACCACTAGAA TACGAGCCAC
7801    AATAAATAAA AGATTTTATT TAGTTTCCAG AAAAAGGGGG
7841    GAATGAAAGA CCCCACCAAA TTGCTTAGCC TGATAGCCGC
7881    AGTAACGCCA TTTTGCAAGG CATGGAAAAA TACCAAACCA
7921    AGAATAGAGA AGTTCAGATC AAGGGCGGGT ACACGAAAAC
7961    AGCTAACGTT GGGCCAAACA GGATATCTGC GGTGAGCAGT
8001    TTCGGCCCCG GCCCGGGGCC AAGAACAGAT GGTCACCGCG
8041    GTTCGGCCCC GGCCCGGGGC CAAGAACAGA TGGTCCCCAG
8081    ATATGGCCCA ACCCTCAGCA GTTTCTTAAG ACCCATCAGA
8121    TGTTTCCAGG CTCCCCCAAG GACCTGAAAT GACCCTGTGC
8161    CTTATTTGAA TTAACCAATC AGCCTGCTTC TCGCTTCTGT
8201    TCGCGCGCTT CTGCTTCCCG AGCTCTATAA AAGAGCTCAC
8241    AACCCCTCAC TCGGCGCGCC AGTCCTCCGA TAGACTGAGT
8281    CGCCCGGGTA CCCGTGTATC CAATAAATCC TCTTGCTGTT
8321    GCA (SEQ ID NO: 8)
```

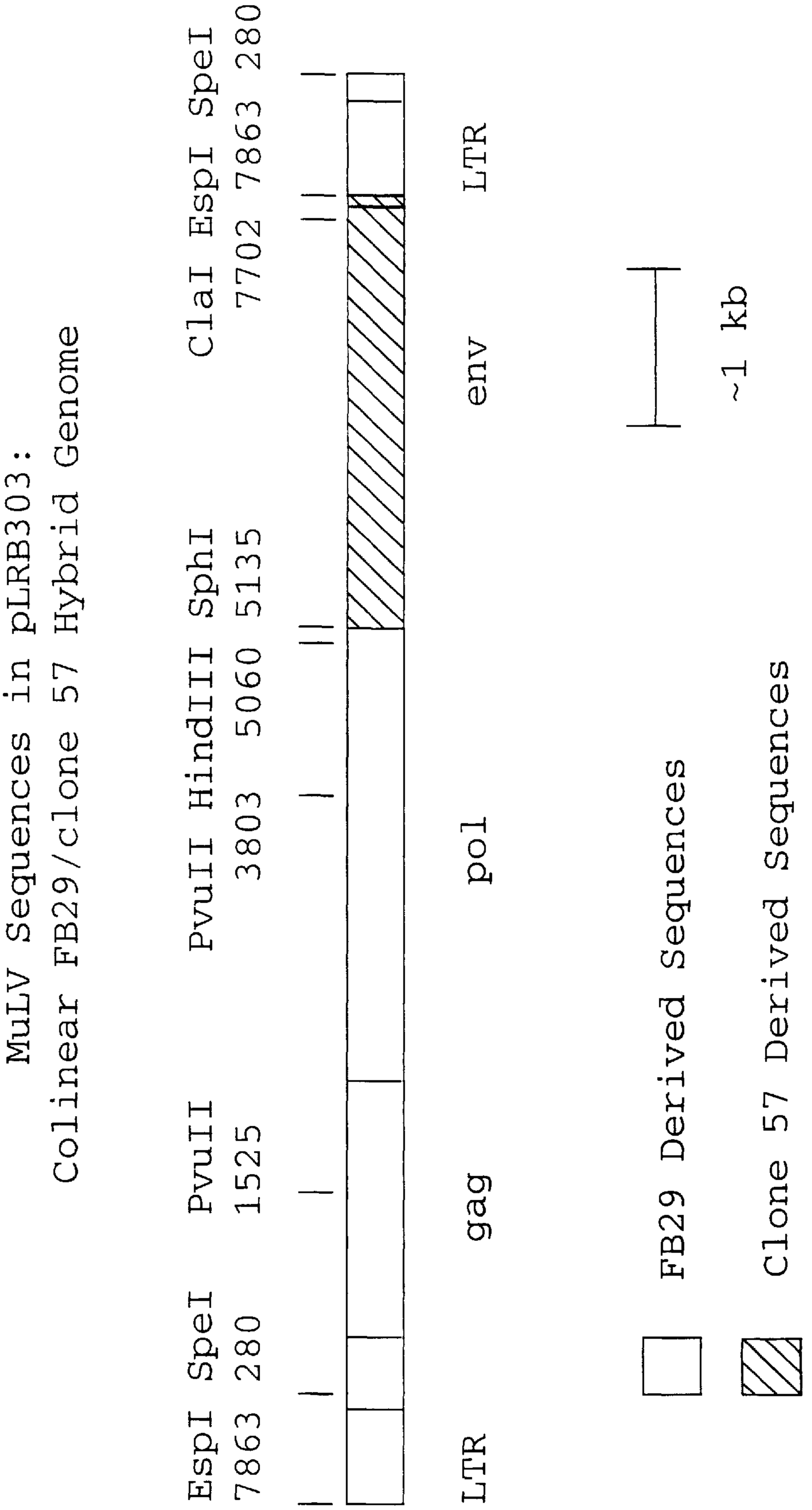

MuLV Sequences in pLRB303:
Colinear FB29/clone 57 Hybrid Genome
EspI SpeI PvuII PvuII HindIII SphI ClaI EspI SpeI
7863 280 1525 3803 5060 5135 7702 7863 280
LTR gag pol env LTR
~1 kb
FB29 Derived Sequences
Clone 57 Derived Sequences

FIG. 2

FIG. 3A

```
                |    10       |    20       |    30       |    40
   1    gaactcgagc agggCTAGTA CAGACACAGG CGCATAAAAT
  41    CAATCAAAGA CACAGGACAA TGGACAGACA CAGAACAATT
  81    GCTGGCCAGC TTACCTCCCG GTGGTGGGTC GGTGGTCCCT
 121    GGGCAGGGGT CTCCAGATCC CGGACGAGCC CCCAAATGAA
 161    AGACCCCCGA GACGGGTAGT CAATCACTCT GAGGAGACCC
 201    TCCCAAGGAA CAGCGAGACC ACGAGTCGGA TGCAACAGCA
 241    AGAGGATTTA TTGGATACAC GGGTACCCGG GCGACTCAGT
 281    CTATCGGAGG ACTGGCGCGC CGAGTGAGGG GTTGTGAGCT
 321    CTTTTATAGA GCTCGGGAAG CAGAAGCGCG CGAACAGAAG
 361    CGAGAAGCAG GCTGATTGGT TAATTCAAAT AAGGCACAGG
 401    GTCATTTCAG GTCCTTGGGG GAGCCTGGAA ACATCTGATG
 441    GGTCTTAAGA AACTGCTGAG GGTTGGGCCA TATCTGGGGA
 481    CCATCTGTTC TTGGCCCCGG GCCGGGGCCG AACCGCGGTG
 521    ACCATCTGTT CTTGGCCCCG GGCCGGGGCC GAAACTGCTC
 561    ACCGCAGATA TCCTGTTTGG CCCAACGTTA GCTGTTTTCG
 601    TGTACCCGCC CTTGATCTGA ACTTCTCTAT TCTTGGTTTG
 641    GTATTTTTCC ATGCCTTGCA AAATGGCGTT ACTGCGGCTA
 681    TCAGGCTAAG CAACTTGGTG GGGTCTTTCA TTCCCCCCTT
 721    TTTCTGGAAA CTAAATAAAA TCTTTTATTT ATCATGGCTC
 761    GTATTCTAGT GGTTTTAGCT GGTGGTATTG TTGAGTCAGG
 801    ACTAAAGCCT GGACTACTGA GATCCTGTCT TTAACAAATT
 841    GAACTAATCG ATtcattagc tagcTCCTGC TGGCGGGGGC
 881    TGAGTGGGAG TGGGGGACGG GGAAATCAAT GTGGGAGTCG
 921    AATTAGAGGC GGGGGGAGAC TTGGCAGGTT TGGGTAGGGG
 961    ATTAGGTCGC GGGAGCGAAA GTTGGTCTGC CAGGACGGGG
1001    TTCGGTCCTA TCGGGACCCG AGGTCCTAGA TTTTGATATC
1041    TGAGTCGGAT CCCGAAAGTA AGCCCCGGGT CCCGCCCAGA
1081    GACATAAAGA CGTAGACCCC AATAGTGTCC AGTTGTCCAT
1121    GAGGTGACCT GTTTCCCGGC GTTTGTAAAC TGGATAGCCA
1161    AGGGATTGCA CCACTTATTG TCTTTGCATA CCTGGACAGC
1201    CTGGCTAGTG GTGAGATTGT TGTCCACTGT GATGTAGTCC
1241    CAAGAGGAGG AGGGCTTCCA GTATACTCTA CCGGTTGTCT
1281    CGCAGCCCCA AGAGGCACAG TAGAAGGAGT CTGGACCTCC
1321    ACAGGACTTG GCTTCCCGGG GGCGATGTGA CCCGGGGCAG
1361    ACATAAAATC CCTCACTTGA TTTATGAGTT ACCTGGTCTA
1401    GCTTAAGTCT GTTCCAGGCA GTGTTGCACC GAGGGGTGAG
1441    GGAGGTCAAG GGCTCGTCGC AGTCTCTGGA ACAGCCTGCA
1481    CTGCTCCCGC TGCTCCCTGA GCAACAAGGG GGCCCCGGGG
1521    GCGAGGAATA GGGGGCCTGA TACTCTAGCC CCCAGTGGGG
1561    CGGCCCACTG AGAGCTAACA TACACAAATC TGGGGTGAGG
1601    ACTGGCCACC AAGTCCACAG AGGGTGGTTG CCTGATATTG
```

FIG. 3B

```
                |    10       |    20       |    30       |    40
1641    CCCATACTGT CTCCCGATCC CCATTGGTCA CTTCCCAGGT
1681    AATGTTGTAG ACCTGGTGAG GGCTGGAGCC GGGTGCTGCG
1721    GATCTGGCCC CTTTGAGAGA CAGGAAGAGA ATTAAGGGGA
1761    TTAGGAGGTC CCGCGGGTCA ATCTTATCTT TAGGGGATTT
1801    TGGGAGCGTT GAACACGCCA TGTCGATTCT GCTGGTGGCT
1841    CAATCCTGGT GTCGGCAGCC TTTACGTGGG CAGCGTGGAT
1881    CCACGCTGCA ATGCCGTCTA CTTTGAGAGC GGTGGGGGTA
1921    GTCAGTAGGA CGGTATAGGG TCCTTTCCAG CGGGGTTCTA
1961    GATTTTTAGT TTGGTGTCTG CGGACCCACA CTGTGTCACC
2001    GACCCGGAAA GGGTGAGGTA CTACCGGCCG GTCTAGTTGC
2041    TCTTGGTAAG CTGCCGCCAA CGGTCTCCAG ACTTCGTGCT
2081    GGACCAGGTA GAGTGCCTGT AAATGAGCTT GGAGAGAGGG
2121    GTTATGAGTA ACCTTTGCCA TGTCAGGATC AGGGAAGTTT
2161    ACAAGGGGCG GGGGTGCCCC ATATAAGATT TCATATGGGG
2201    TGAGACCGTG GGGGCCCGGC GTGTTGCGGG CTCGATACAG
2241    GGCAAGGGGA AGCAGGAGCA CCCAGTCCCT AGAGCCAGTT
2281    GCAAGCGTCA ATTTAGTTAA AGTCTCCTTG ATTGTCCTAT
2321    TCATTCTTTC TACCTGACCT GAACTCTGGG GTCTGTAAGC
2361    ACAATGTAGT TTCCAATCAA CCCCCAATAA ATCGGCTACT
2401    GTCTGACTTA CCTTGGAGAC GAAGGCAGGC CCATTGTCGG
2441    TTCCCAATAC CTGTGGCATG CCGAATCTGG GGAAGATTTC
2481    TTCTAGTAGC TTCTTGGTTA CAACTTTGGC AGTTTCTTTC
2521    TTGGTTGGGA AAGCTTCTAC CCATCCAGAG AAAGTGTCTA
2561    TGAAAACTAA AAGATATTTA TACCCATACA GGCCAGGTTT
2601    TACCTCAGTG AAATCAATTT CCCAGTGGGT GCCGGGTCGG
2641    TGCCCTCGAA CTCTAGTCCC TTGTTTGACG GCAGACTTGC
2681    TGGCATTGAC CTGTGCACAG GCTTGGCAAG TCTCAGTGAT
2721    GTCTTTGAGC GTTCGATCCC GGTTCAGCAT GTAATAAGGA
2761    CAGTAGTTCC TTTCTAGAAG AGCCTTTGTT TTTGAGAAAC
2801    TGAGGTGGGT CAATTGATGA AGAAAATCTA ATAGTTCAAA
2841    GGTGAATTGA TCAGGCATTA CAGGCTTTCC CTGATAAACC
2881    CAACACTTCT TTGCATCGTC ATAAGTGGCC CCTAGTTTAG
2921    TCAGATCTTT TATGTCAGTC ACCGTATAGT GAAAATGTTC
2961    ATGAGTATAG GGGGCTGAAT TTTCTATCAG AAGTGTGGAA
3001    GTCTCTGGAG TTTCTCTAGT GGCTACTTCT CGGGCCGCTT
3041    GGTCGGCCAT CCTGTTGCCC CTTGCCTCCG CGCGGTTTCC
3081    CTTCTGATGT CCCGGGCAAT GAATTATGCT AAGTCTTTTG
3121    GGCAGGAAGA GAGCCTTCAG TAGGGCCAAG ATCTCGTCCT
3161    TATTTTTGAT TTCTTTTCCT TCTGATGTGA GCAACCCGCG
3201    CCTTCTATAT ATTTCTCCGT GAATATGGGC AGTGGCAAAA
3241    GCATAACGGC TATCGGTGTA AACATTCAGC TTCTTACCTT
3281    CTGCCATTTT TAAGGCTTGG GTGAGCGCTA TCAACTCAGC
3321    TCTTTGGGCC GATGTCCCGG CTGGCAGTGC TTTGGCCCAG
```

FIG. 3C

```
                |    10       |    20       |    30       |    40
3361    ACTACCTCGG TCTCGGTGGT TACTGCTGCT CCGGCCTTGC
3401    GCTGCCCCTC TTGCAGGAAG CTGCTCCCAT CTGTGTACCA
3441    GGTGTGGTCA GCGTCTGGGA GAGGCTGGTC CGTAAGATCT
3481    GGTCTAGTTC CGTGGGCTTC AGCCAAGATG TCAAGGCAGT
3521    CATGTTGCAG CCCCTCCTCA GGTAGAGGGA GCAGCGTAGC
3561    TGGGTTTAGG GCCACTATTG GTCCGAACTG GACTCGGTCC
3601    GTGTCCAGAA GCAGAGCCTG GTAGTGGGTC ATTCGGGCGT
3641    TGGAGAGCCA GCGATCAGGG GGTTGCTTAA CTAGTGCCTC
3681    TACTGCATGG GGGGCCAGAA TGACTAGTGG CTGTCCCATG
3721    GTGAGCTTGC CAGCGTCTTT GGTCAGAACG GCGATGGCTG
3761    CTACCATCCG TAGGCAAGGG GGCCACCCAG CTGCCACTGG
3801    GTCTAGCTTT TTGGACAGGT AGGCCACCGG CCGACGCCAA
3841    GGCCCCAGTT TTTGCGTTAG GACACCTTTG GCGTAGCCCT
3881    GCTTCTCGTC AACAAAAAGT TCGAAGGGCT TAGTCAAGTC
3921    TGGCAATCCC AGGGCAGGGG CAGTTAAGAG AGCCTGCTTG
3961    ATCTCTTGGT AGGCCTTTTG CTGGTCTGGG CCCCACTCAA
4001    ACAGAGTCCC CGTTTTGGTG AGAGGGTACA AGGGGGCTGC
4041    CATTTCTGCA AACCCAGGGA TCCAGAGGCG ACAGAAGCCT
4081    GCCGTCCCTA GGAACTCCCT TAGTTGTCGA GGGGTCTTCG
4121    GAGTAGGCTG CCCCATCACA GTCTCTTTTC TGGCCTCAGT
4161    CAGCCATCTC TGACCCTCTT TTAGAAGATA CCCCAGATAC
4201    TTGACCTGTT TCTGGCAAAT TTGGGCTTTC TTGGCCGAGG
4241    CCCGATATCC GAGGTCCCCT AGGGTTTGTA ACAGGGCCCG
4281    CGTACCTTGT TGACAGTCAA GCTCAGAAGT GGCGGCCAGC
4321    AGTAAGTCAT CTACATACTG GAGCAGAATC AGGTCTGGGT
4361    GCTGGATCCG GAAGTCTGCG AGGTCCCTGT GCAGGGCTTC
4401    ATCAAACAGG GTGGGACTGT TTTTGAAACC CTGCGGGAGT
4441    CTGGTCCAGG TTAATTGTCC TGAGATTCCC ATCTCTGGAT
4481    CTCTCCACTC AAAGGCGAAG AGAGACTGAC TGGTGGGGTG
4521    GAGTCTCAGG CAGAAAAAAG CATCTTTTAA GTCAAGCACA
4561    GTGTACCACT GGTGGGACGG TGGGAGCCCG CTCAAGAGGT
4601    TGTAAGGGTT GGGCACGGTG GGGTGGATGT CTTCCACCCG
4641    CTTGTTGACT TCTCTCAGAT CCTGGACAGG CCTATAATCA
4681    TTAGTCCCCG GTTTCTTAAC GGGTAGCAGG GGCGTGTTCC
4721    AGGGGGACTG GCAGGGTACC AGAATTCCCT GATCCAGCAG
4761    TCTCTGTATG TGGGGCTTGA TCCCCAGTCT GGCTTCTTGT
4801    GACATGGGGT ATTGTTTTAT GGACACGGGG GTAGAGGTTG
4841    CCTTCAGAGG TATGATCAGA GGAGCTTGGC GAACGGCCAG
4881    CCCCATGCCC CCGGTTTCTG CCCAGGCCTG GGGAAAATCA
4921    GAGAGCCATG TGGACCCTAG AGGCACATCT GGCCCTTTTG
4961    AGGTCTCATG TAGCCGATAC TCATCTTCTA TGTTTAGGGT
5001    CAGCACTTGC AGGGGCTGTC CCATTGGTCC CACAACCTGA
5041    GCTCCTGATC CCTCAAAGTG AATTTGGGCT TTTAGTTTAG
5081    TCAGCAAATC TCTTCCTAGC AGAGGATAGG GGCAATCTGG
```

FIG. 3D

```
          |   10      |   20      |   30      |   40
5121   TACATGGAGG AAAGAATGGG TGACCTTACC GGTGGCTAGG
5161   TGCACTCGGC GATCCGTGGT CCAGCGATAC CGCTTCCCTC
5201   CAGTAGCCCC TTGGACCCAG GCAGACTTGT CACTTAGGGG
5241   TCCAGGATTT TGGGTCAGCA CGGAGTGTTG GGCCCCAGTA
5281   TCCACTAGGA AGGTGACGGG TTGCCCCCCG ACTCTGAGGG
5321   TTATCCTGGG TTCAGGGGGG GGCTCCTGAC CCTGACCTCC
5361   CTAATCGTCT AAGGTCAGGA GGGAGGCCTG GGGTCGTGGT
5401   CCCCGGGGTC CTCTTGGCTT CTTGGGGCAA TCTCTAGCCC
5441   AATGTCCCTT TTCTTTGCAG TAGGCACACT GGTCGTGGTC
5481   GAGTTGGGGC CTCCTTCGCT CTCCTCCCTG TCTATCCTGT
5521   CTCTGCCCGC TAACGACAGT AGCCAGCAAC TTACTCATTT
5561   CTCTATGTCT TCTGCGGTCC CTCTCCTTCT CTCTCTGCAC
5601   ATCCTCTGCC CTACGGCGTT CTTCCTTTTC CTCTGTTTCT
5641   CTCCTAATAC GTTCCTCTCT TTCTTCCGGG GTTTCTCGTT
5681   TATTAAAGAT CTTTTCAGCT TCCCTCACTA AGTCTCCTAA
5721   GGTCTTACTC TTCAAATCTT CTAACCGCTC TAACTTTCGC
5761   CCGATATCCG GGGCGGACTG CCAGATGAAT GACATGGCCA
5801   CATTGGTTTC TTGCCCTGGG TCCTCAGGGT CATAAGGAGT
5841   GTATCTGCGA TAGGCCTCCT TGAGTCTCTC TAAAAAGGCT
5881   GAGGGAGACT CATTAGGTCC CTGGGTTATC CCTTTTACCT
5921   TGGCCAAATT GGTGGGGCTT CTGCCCGCGT TTTGGAGACC
5961   CGCTAGGAGC AACTGGCGAT AGTGGACTAG GTGGTTCCTA
6001   CCTCGTTGGG TGTTGTAGTC CCAGTCGGGA CGTTCCAAGG
6041   GAAAAGCATC ATTAATGTCA TTGGGCAGCT GAGTTGGGCG
6081   TCCGTCCTCC CCTCGAACCG CCTTTCGGGC CTCTAGGAGC
6121   ACTCGCTGTT TTTCTTCTCC CGTCAGCAGG GTCCCTAATA
6161   GCTGTTGGCA GTCATCCCAA GTGGGCTGAT GAGTAAGGAG
6201   AACGGACTCG ATCAAAGCTG TCAATTTAGC TGGGTCCTCG
6241   GAGAAAGAGG GGTTGTTATT TTTCCAGTTA TAGAGGTCAG
6281   AGGAGGAAAA TGGCCAGTAT TGATACTGTC CATTCCCTCC
6321   CAGGCGAAGG GGGAACGCCT GAGAGGTAGT AGAATCCGCC
6361   ACGGGGGGTT CTTTTCTTCC CCGCAGGCGG GATACCATTG
6401   GGGAAGGGTC AGGGGCTCCT TCTGTAGGGG CCACTTCTCC
6441   GCTATCGCCG TTCCCGTCAG GAGAGGGTGG CCCTGGGTCC
6481   CGGTAAGGCG GAGGGTCCTC CGTGAGTAGA TCAATGAGTG
6521   GTCCTCCGCT ATCAGGAAGG ACTTGAGGCC TAGGTTTGGT
6561   GTTTAAAGGA GAAGTGAGAG CCGGATAGAG GGAGGACTGG
6601   GGCGGGGTCG AGAGTGGGGG TTCAGGTGGG AGAGAGGGGG
6641   CTGAAGGGGG AAGAGAGAGG GGAGGTTTAG GGTGCACGAA
6681   GGGTCTGACC CAGGGAGGGG GGTCTACTGC TATAGCTTCC
6721   CAGGTCACGA TGTAGGGGAC CTGATCCGGA TGTCCATGTG
6761   GGCCAGGTGA GAAGACCTTG ATCTTAACCT GTGTAATAAT
6801   GTCTGGGTTA AAAGTGCCGT CTCGTGGCCA TCCGACGTTG
6841   AAGGTTGGCC ATTCTGCAGA GCAGAATGTA ACCCAGCGCC
```

FIG. 3E

```
          |    10       |    20       |    30       |    40
6881    TTTTTCTAAC CTCTACCGAC AGGTTGTGGG CTGTCCGTTC
6921    GACATCCTTC CAGTGGTCTA AAGTCAAACT TAAGGGGGTG
6961    GTAACAGCCT GGCCCATGTT TTCAGACAAA TACAGAAAAA
7001    CAGTCAAACA GAGACAACAC AGAACGATGC TGCAGCAGAC
7041    AAGACGCGCG GCGCGGCTTC GGTTCCAAAC CGAAAGCAAA
7081    AACTCAGACG GGGGCGGAAA CCGTTTTAGC CCTCTGTCTC
7121    CTACCAGAAC CACATACCCC TCCTCTAAGG GGGGTGCACC
7161    AAAGAGTCCA AAACGATCGG GATGGTTGGA CTCTGGCCGG
7201    GCCACAAAAA TGGCCCCCGA AGTCCCTGGG ACGTCTCCCA
7241    GGGTTGCGGC CGGGTGTCTC GAACTCGTCA GTTCCACCAC
7281    GGATCCGCCA GATACCAATC TAGTCGGCCA ACTAGTACAG
7321    ACACAGGCGC ATAAAATCAA TCAAAGACAC AGGACAATGG
7361    ACAGACACAG AACAATTGCT GGCCAGCTTA CCTCCCGGTG
7401    GTGGGTCGGT GGTCCCTGGG CAGGGGTCTC CAGATCCCGG
7441    ACGAGCCCCC AAATGAAAGA CCCCCGAGAC GGGTAGTCAA
7481    TCACTCTGAG GAGACCCTCC CAAGGAACAG CGAGACCACG
7521    AGTCGGATGC AACAGCAAGA GGATTTATTG GATACACGGG
7561    TACCCGGGCG ACTCAGTCTA TCGGAGGACT GGCGCGCCGA
7601    GTGAGGGGTT GTGAGCTCTT TTATAGAGCT CGGGAAGCAG
7641    AAGCGCGCGA ACAGAAGCGA GAAGCAGGCT GATTGGTTAA
7681    TTCAAATAAG GCACAGGGTC ATTTCAGGTC CTTGGGGGAG
7721    CCTGGAAACA TCTGATGGGT CTTAAGAAAC TGCTGAGGGT
7761    TGGGCCATAT CTGGGGACCA TCTGTTCTTG GCCCCGGGCC
7801    GGGGCCGAAC CGCGGTGACC ATCTGTTCTT GGCCCCGGGC
7841    CGGGGCCGAA ACTGCTCACC GCAGATATCC TGTTTGGCCC
7881    AACGTTAGCT GTTTTCGTGT ACCCGCCCTT GATCTGAACT
7921    TCTCTATTCT TGGTTTGGTA TTTTTCCATG CCTTGCAAAA
7961    TGGCGTTACT GCGGCTATCA GGCTAAatca gatctgccgg
8001    tctccctata gtgagtcgta ttaatttcga taagccaggt
8041    taacctgcat taatgaatcg gccaacgcgc ggggagaggc
8081    ggtttgcgta ttgggcgctc ttccgcttcc tcgctcactg
8121    actcgctgcg ctcggtcgtt cggctgcggc gagcggtatc
8161    agctcactca aaggcggtaa tacggttatc cacagaatca
8201    ggggataacg caggaaagaa catgtgagca aaaggccagc
8241    aaaaggccag gaaccgtaaa aaggccgcgt tgctggcgtt
8281    tttccatagg ctccgccccc ctgacgagca tcacaaaaat
8321    cgacgctcaa gtcagaggtg gcgaaacccg acaggactat
8361    aaagatacca ggcgtttccc cctggaagct ccctcgtgcg
8401    ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc
8441    gcctttctcc cttcgggaag cgtggcgctt tctcaatgct
8481    cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc
8521    caagctgggc tgtgtgcacg aacccccccgt tcagcccgac
8561    cgctgcgcct tatccggtaa ctatcgtctt gagtccaacc
8601    cggtaagaca cgacttatcg ccactggcag cagccactgg
```

FIG. 3F

```
          |   10      |   20      |   30      |   40
8641   taacaggatt agcagagcga ggtatgtagg cggtgctaca
8681   gagttcttga agtggtggcc taactacggc tacactagaa
8721   ggacagtatt tggtatctgc gctctgctga agccagttac
8761   cttcggaaaa agagttggta gctcttgatc cggcaaacaa
8801   accaccgctg gtagcggtgg ttttttgtt tgcaagcagc
8841   agattacgcg cagaaaaaaa ggatctcaag aagatccttt
8881   gatcttttct acggggtctg acgctcagtg gaacgaaaac
8921   tcacgttaag ggattttggt catgagatta tcaaaaagga
8961   tcttcaccta gatcctttta aattaaaaat gaagttttaa
9001   atcaatctaa agtatatatg agtaaacttg gtctgacagt
9041   taccaatgct taatcagtga ggcacctatc tcagcgatct
9081   gtctatttcg ttcatccata gttgcctgac tccccgtcgt
9121   gtagataact acgatacggg agggcttacc atctggcccc
9161   agtgctgcaa tgataccgcg agacccacgc tcaccggctc
9201   cagatttatc agcaataaac cagccagccg gaagggccga
9241   gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag
9281   tctattaatt gttgccggga agctagagta agtagttcgc
9321   cagttaatag tttgcgcaac gttgttgcca ttgctacagg
9361   catcgtggtg tcacgctcgt cgtttggtat ggcttcattc
9401   agctccggtt cccaacgatc aaggcgagtt acatgatccc
9441   ccatgttgtg caaaaaagcg gttagctcct tcggtcctcc
9481   gatcgttgtc agaagtaagt tggccgcagt gttatcactc
9521   atggttatgg cagcactgca taattctctt actgtcatgc
9561   catccgtaag atgcttttct gtgactggtg agtactcaac
9601   caagtcattc tgagaatagt gtatgcggcg accgagttgc
9641   tcttgcccgg cgtcaatacg ggataatacc gcgccacata
9681   gcagaacttt aaaagtgctc atcattggaa aacgttcttc
9721   ggggcgaaaa ctctcaagga tcttaccgct gttgagatcc
9761   agttcgatgt aacccactcg tgcacccaac tgatcttcag
9801   catcttttac tttcaccagc gtttctgggt gagcaaaaac
9841   aggaaggcaa aatgccgcaa aaaagggaat aagggcgaca
9881   cggaaatgtt gaatactcat actcttcctt tttcaatatt
9921   attgaagcat ttatcagggt tattgtctca tgagcggata
9961   catatttgaa tgtatttaga aaaataaaca aatagggggtt
10001  ccgcgcacat ttccccgaaa agtgccacct gacgtctaag
10041  aaaccattat tatcatgaca ttaacctata aaaataggcg
10081  tatcacgagg ccctttcgtc tcgcgcgttt cggtgatgac
10121  ggtgaaaacc tctgacacat gcagctcccg gagacggtca
10161  cagcttgtct gtaagcggat gccgggagca gacaagcccg
10201  tcagggcgcg tcagcgggtg ttggcgggtg tcggggctgg
10241  cttaactatg cggcatcaga gcagattgta ctgagagtgc
10281  accatatgga catattgtcg ttagaacgcg gctacaatta
10321  atacataacc ttatgtatca tacacatacg atttaggtga
10361  cactata (SEQ ID NO: 9)
```

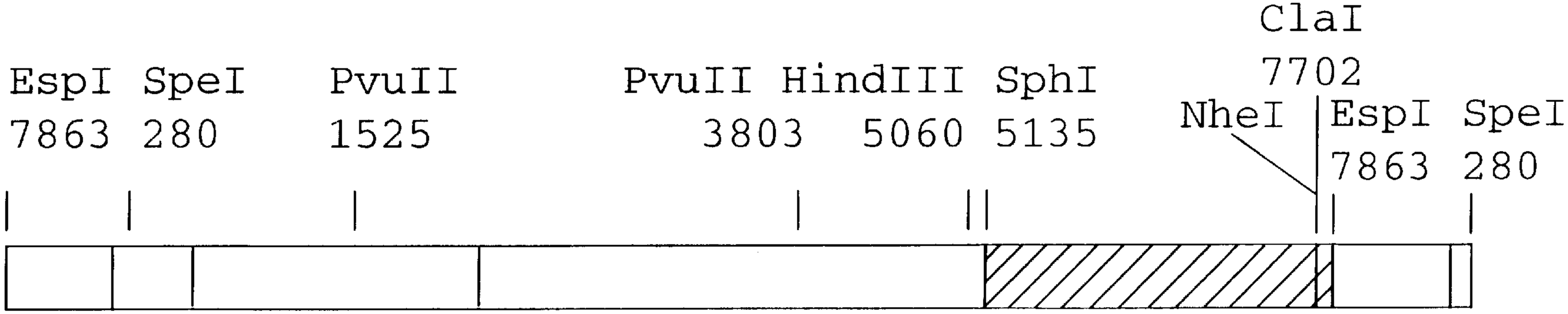
```
          |    10       |    20       |    30       |    40
  1   MACSTLPKSP KDKIDPRDLL IPLILFLSLK GARSAAPGSS
 41   PHQVYNITWE VTNGDRETVW AISGNHPLWT WWPVLTPDLC
 81   MLALSGPPHW GLEYQAPYSS PPGPPCCSGS SGSSAGCSRD
121   CDEPLTSLTP RCNTAWNRLK LDQVTHKSSE GFYVCPGSHR
161   PREAKSCGGP DSFYCASWGC ETTGRVYWKP SSSWDYITVD
201   NNLTTSQAVQ VCKDNKWCNP LAIQFTNAGK QVTSWTTGHY
241   WGLRLYVSGR DPGLTFGIRL RYQNLGPRVP IGPNPVLADQ
281   LSLPRPNPLP KPAKSPPASN STPTLISPSP TPTQPPPAGA
321   SZZ (SEQ ID NO: 10)
```

FIG. 3G

```
          |    10       |    20       |    30       |    40
  1   AAPGSSPHQV YNITWEVTNG DRETVWAISG NHPLWTWWPV
 41   LTPDLCMLAL SGPPHWGLEY QAPYSSPPGP PCCSGSSGSS
 81   AGCSRDCDEP LTSLTPRCNT AWNRLKLDQV THKSSEGFYV
121   CPGSHRPREA KSCGGPDSFY CASWGCETTG RVYWKPSSSW
161   DYITVDNNLT TSQAVQVCKD NKWCNPLAIQ FTNAGKQVTS
201   WTTGHYWGLR LYVSGRDPGL TFGIRLRYQN LGPRVPIGPN
241   PVLADQLSLP RPNPLPKPAK SPPASNSTPT LISPSPTPTQ
281   PPPAGASZZ (SEQ ID NO: 11)
```

FIG. 3H

MuLV Sequences in pLRB332:
gp(1-285) gp70 Gene Fragment in
Colinear FB29/clone 57 Hybrid Genome



FB29 Derived Sequences

Clone 57 Derived Sequences

~1 kb

FIG. 4A

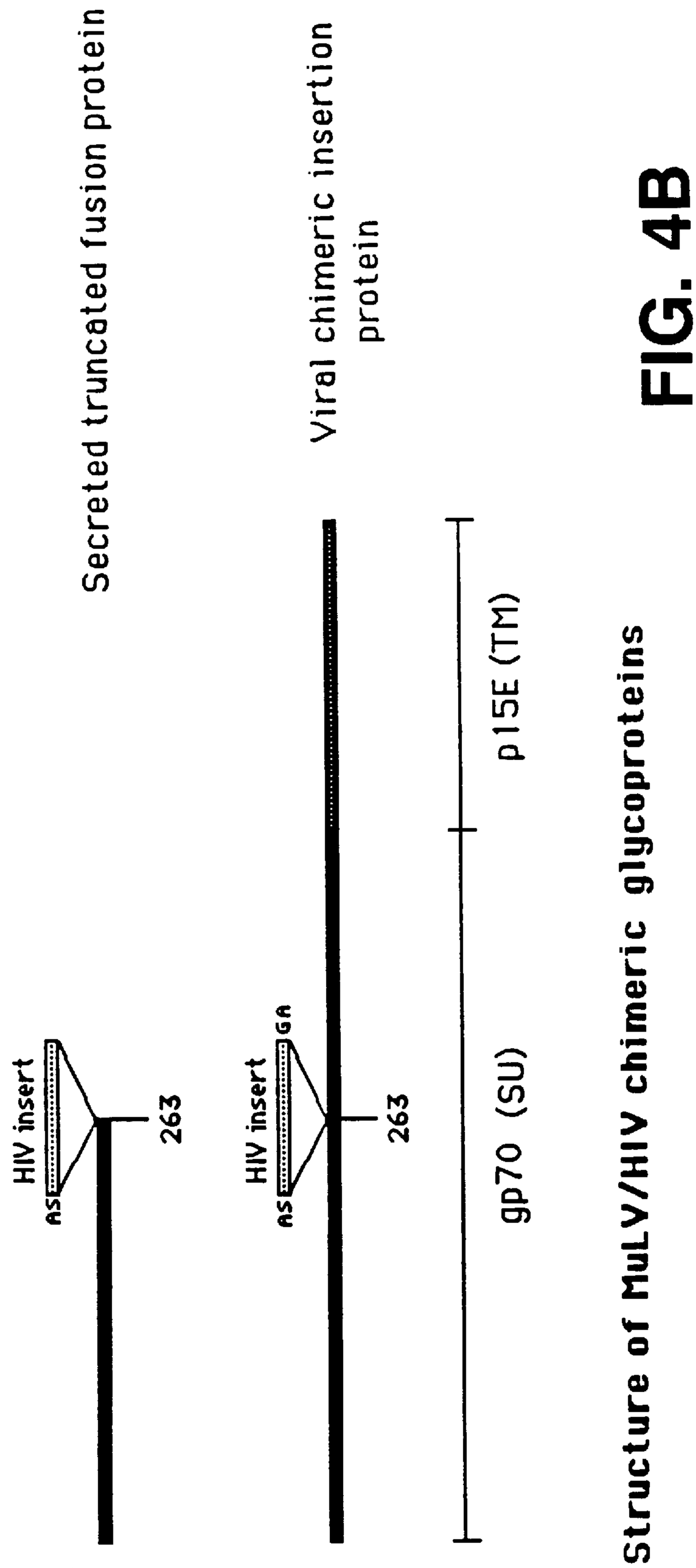

Secreted truncated fusion protein
Viral chimeric insertion protein
B
p15E (TM)
gp70 (SU)
HIV insert
AS
GA
263
HIV insert
AS
GA
263
Structure of MuLV/HIV chimeric glycoproteins

FIG. 4

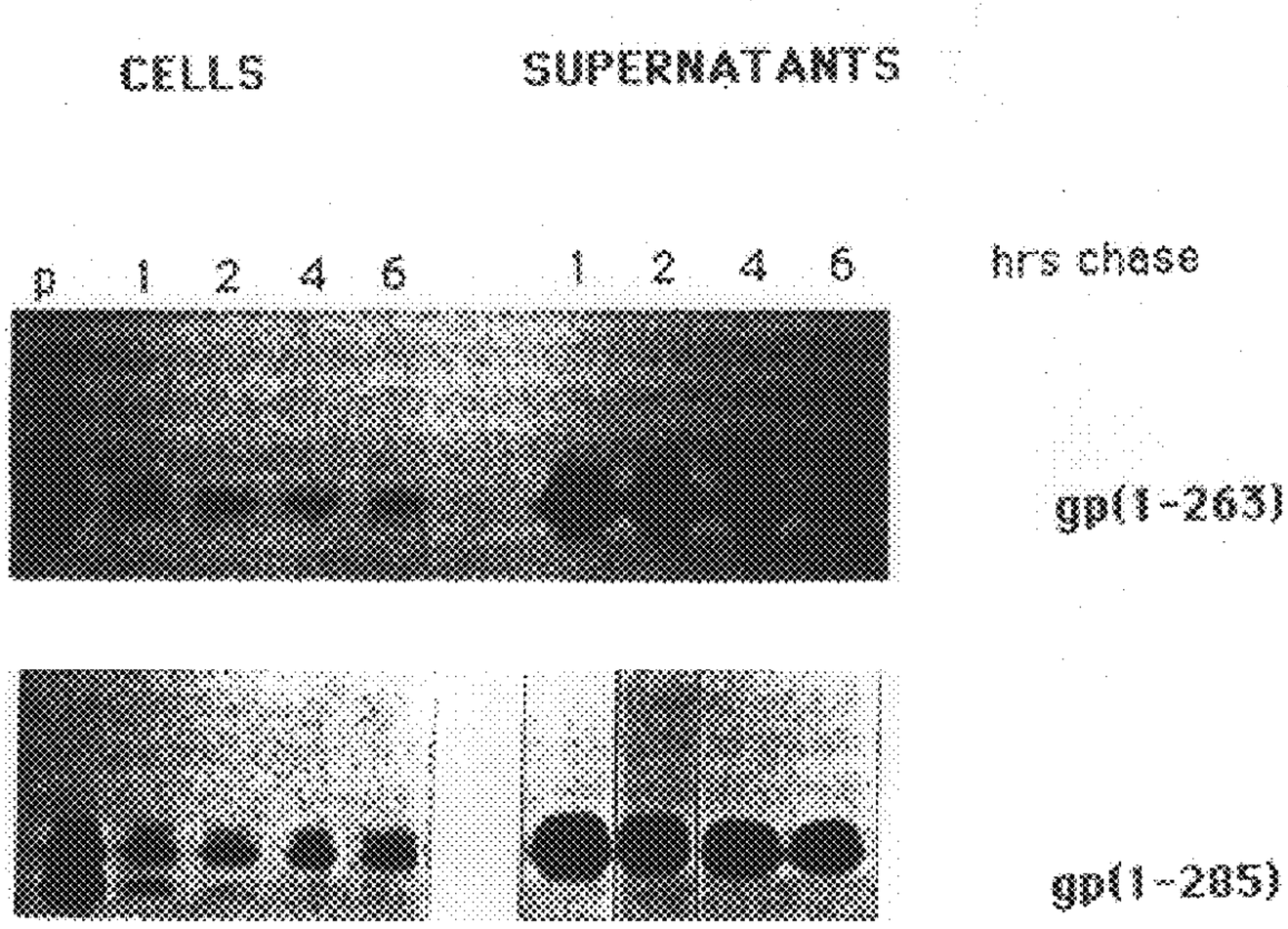
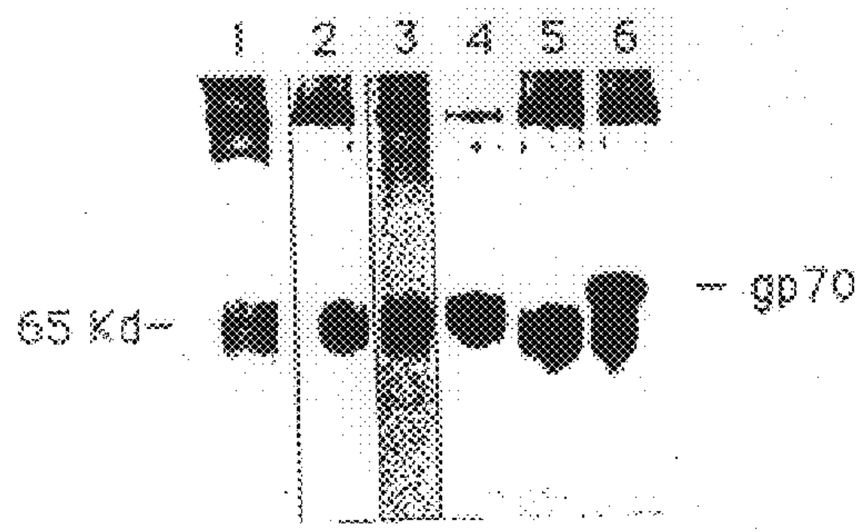


FIG. 5



1. CRA-3
2. C108G
3. G3-4
4. chimp serum
5. MAb 273 (αgp70)
6. goat αgp70 serum

FIG. 6B

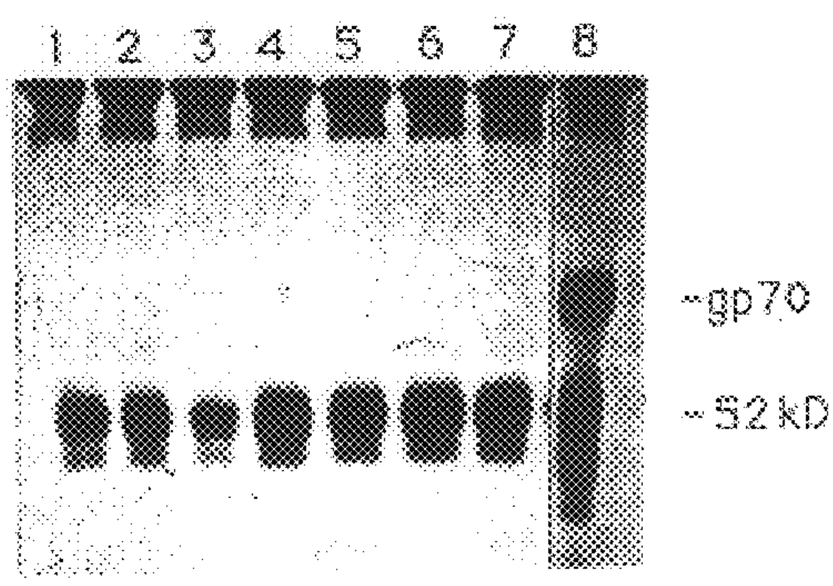
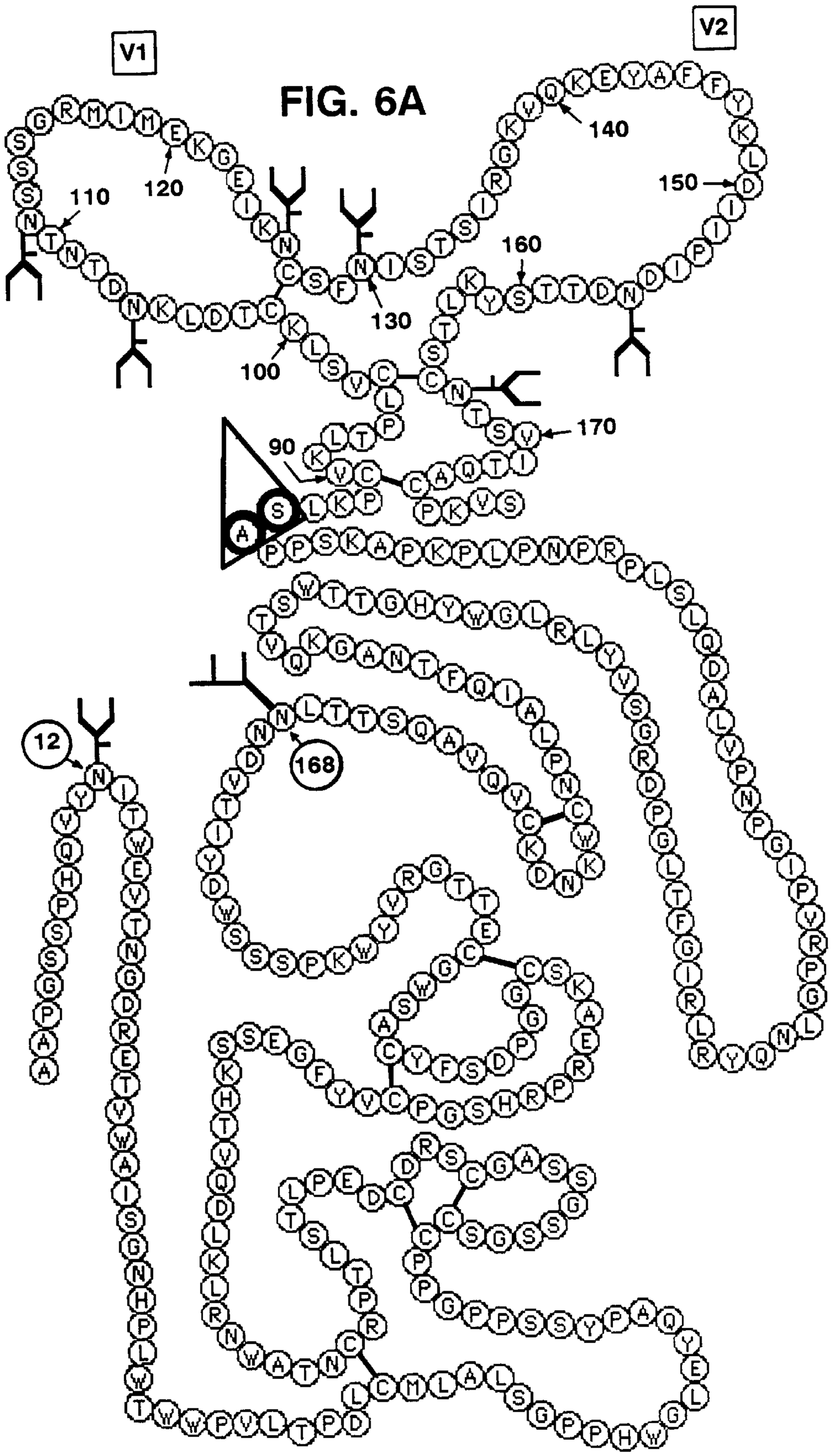


FIG. 10B

FIG. 6A



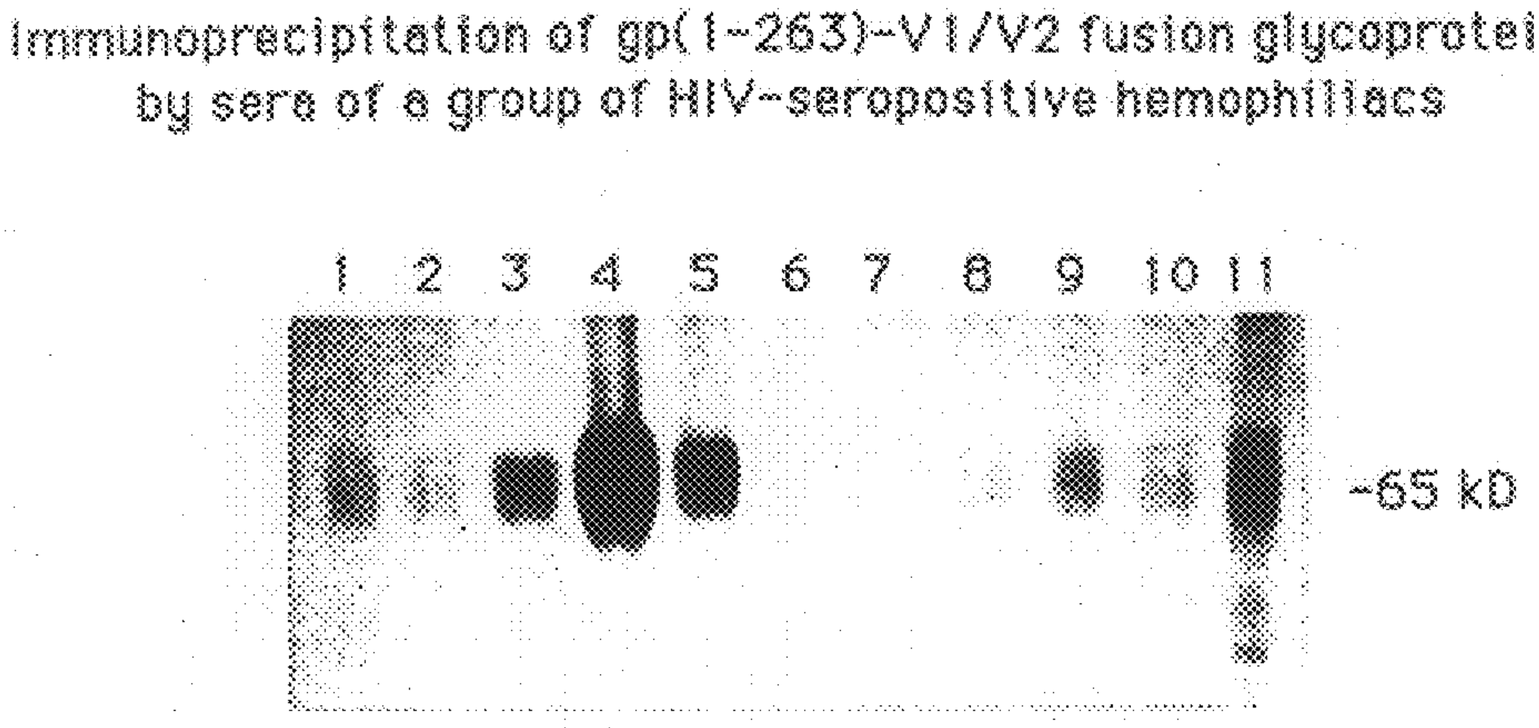


FIG. 7

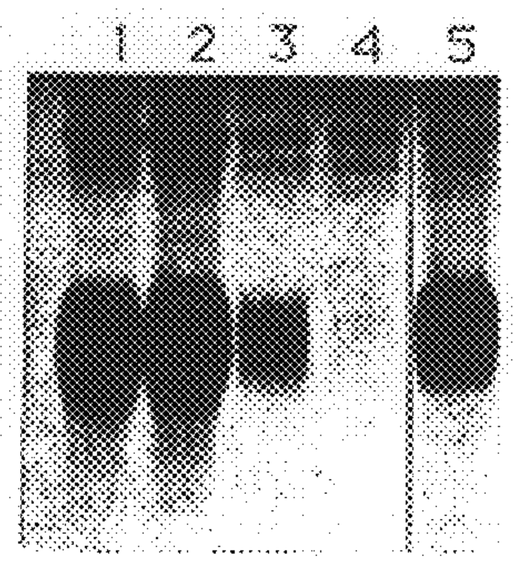


FIG. 8

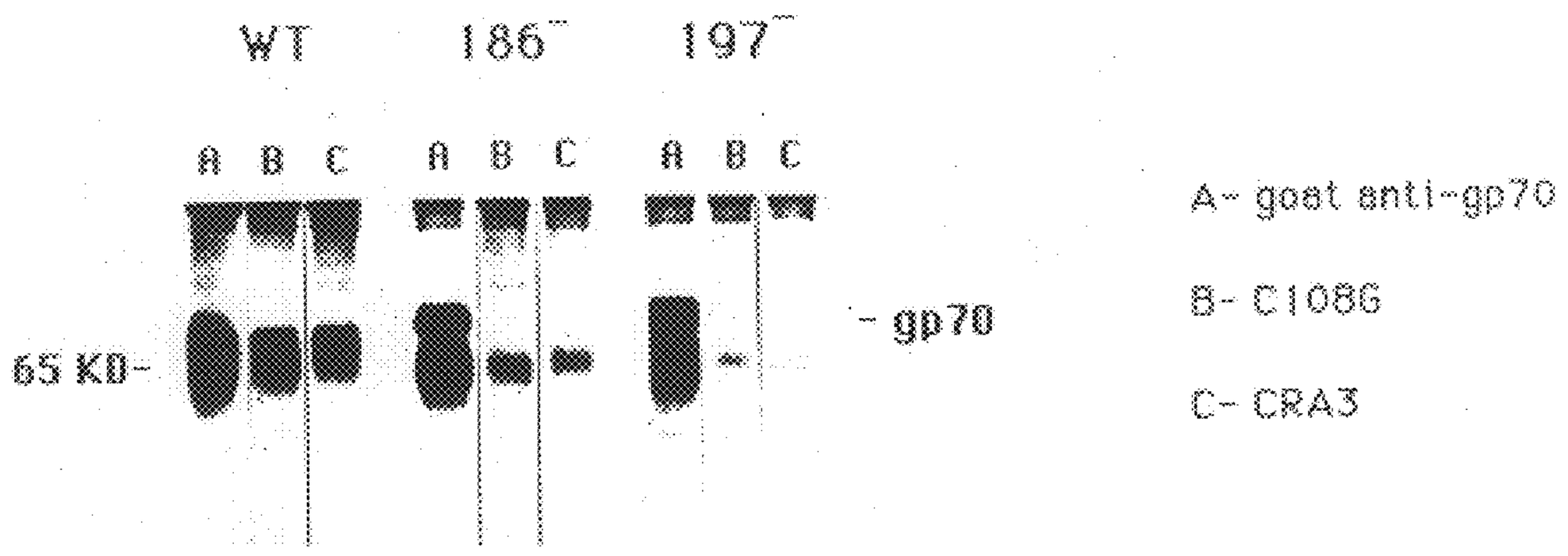
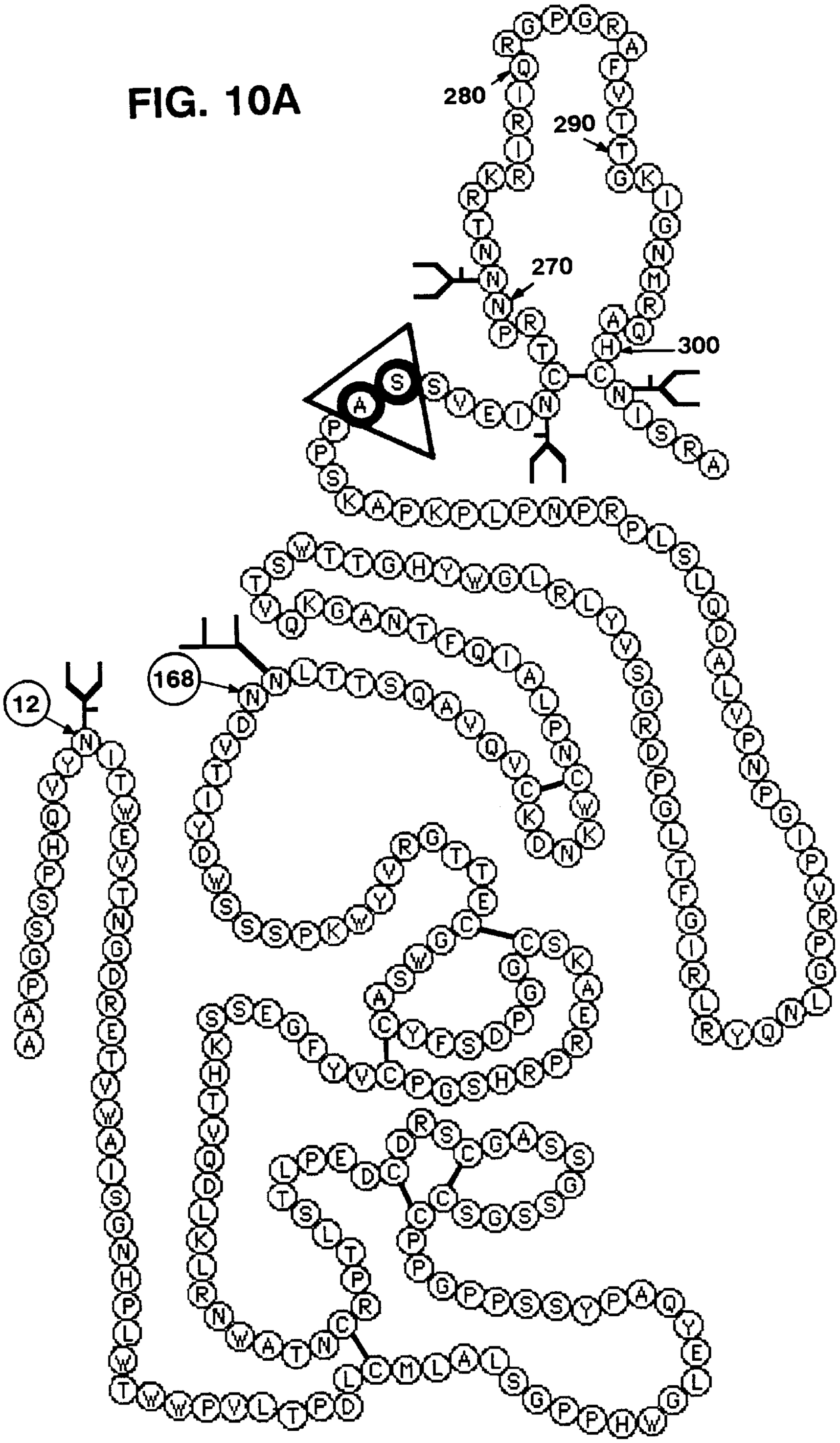


FIG. 9

FIG. 10A



FUSION GLYCOPROTEINS

This is a divisional of application Ser. No. 08/110,300 filed on Aug. 20, 1993 now U.S. Pat. No. 5,643,756, which is a continuation in part of Ser. No. 07/938,100, filed Aug. 28, 1992 now abandoned.

BACKGROUND OF THE INVENTION

It has been shown for retroviral, influenza, and herpes viral glycoproteins that removal of N-linked glycans dramatically reduces immune reactivity (Alexander and Elder, 1984, Science, 226:1328–1330; Sjobloom et al., 1987, J. Gen. Virol. 68:549–554; Olofsson et al., 1990, J. Virol. 71:889–895). Considerable evidence has also accumulated suggesting that N-linked glycosylation is necessary for proper immunoreactivity or immunogenicity of human immuno-deficiency virus (HIV) gp120. For example, an extensive study comparing the immunogenicity of native glycosylated gp120 to that of env 2–3, a nonglycosylated form of the protein produced in yeast, was performed in baboons (Haigwood et al, 1992, J. Virol. 66:172–182). In this study, glycosylated gp120 induced high titers of neutralizing antibodies against the homologous and related viruses, and weak neutralizing titers against more distant viruses, while the nonglycosylated protein yielded only low neutralizing titers directed solely against the homologous virus. In addition, whereas the glycosylated protein was able to bind to CD4, the nonglycosylated protein was not. Other studies have shown that removal of N-linked glycans from native or recombinant gp120 reduces or abolishes binding activity of gp120 to CD4 and diminishes infectivity of HIV-1 (Matthews et al, 1987, PNAS 84:5424–5428; Fenouillet et al, 1989, J. Exp. Med. 169:807–821; Fenouillet et al, 1990, J. Virol. 64:2841–2848). Recent data show that removal of specific carbohydrates from recombinant gp160 reduced both its immunoreactivity with hyperimmune antisera and its immunogenicity in rabbits (Benjouad et al, 1992, J. Virol. 66:2473–2483). Finally, the epitopes of strongly neutralizing MAbs that have been isolated are destroyed by removal of N-linked glycans from the viral proteins (Fung et al, 1992, J. Virol. 66:848–856). These results demonstrate an important role for N-linked glycans in gp120 immunoreactivity and immunogenicity. These glycans may be acting either indirectly, by achieving the correct conformation of gp120, or directly by determining the formation of immunoreactive or immunogenic sites.

A major difficulty in the production of isolated gp120 domains is the fact that all of these domains are highly glycosylated. Considerable evidence suggests that glycans are needed either to achieve correct conformations or for the formation of the epitopes themselves.

The invention described here is intended to address this problem, but can also be used for the expression of non-gp120 glycopeptides whose immunogenicities or biological activities are dependent on correct glycosylation or conformation.

SUMMARY OF THE INVENTION

The present invention relates to vectors for expressing a glycosylated protein in cells, wherein the vector is adapted to express a fusion glycoprotein. The fusion glycoprotein contains an N-terminal fragment of env surface protein of a retrovirus, the surface protein containing a hydrophobic glycosylation signal located about seven residues N-terminal to a Cys-trp-leu-cys sequence (SEQ ID NO:18) located approximately centrally in the surface protein. The

N-terminal fragment includes the N-terminal globular domain of the env surface protein. A selected glycopeptide is fused to the C-terminus of that N-terminal fragment. The selected glycopeptide preferably includes a domain that is glycosylation dependent, a domain that is conformationally dependent, or both. The domain may contain, for example, an immunoreactive or immunogenic epitope, or have another biological activity, such as receptor binding or hormone binding activity.

Also included in the invention are plasmids that encode the above vectors. These plasmids can be used to engineer and express the vectors.

Mammalian cell cultures that produce the novel fusion glycoproteins, the novel fusion glycoproteins, and viral particles incorporating those glycoproteins in their envelope moieties are also included in the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the DNA and amino acid sequence (SEQ ID NO:8) of the FB29 isolate of Friend ecotropic MuLV.

FIG. **2** shows a restriction map of a FB29/clone 57 hybrid.

FIGS. **3**A—F shows the DNA sequence (SEQ ID NO:9) of pLRB332. MuLV sequences (non-coding strand) are shown in upper case; non-MuLV sequences are shown in lower case. The NheI site and stop codons at 864, the first codon of gp70 at 1719, and the env initiation codon at 1821, are underlined.

FIGS. **3**G—H shows the amino acid sequence (SEQ ID NO:10–11) of amino acid 1-285 truncation fragment of gp70 expressed by pLRB332. The terminal ASZZ is the amino acid sequence encoded by the NheI site and two stop codons. The top sequence (SEQ ID NO:10) includes the signal peptide, and the bottom sequence (SEQ ID NO:11) does not.

FIG. **4**A shows a restriction map of the MuLV regions of pLRB332.

FIG. **4**B shows a diagram of truncation and insertion fusion glycoproteins described in the examples.

FIG. **5** shows autoradiograms of an SDS-PAGE analysis of immunoprecipitated gp(1-263) and gp(1-285) truncation glycoproteins described in the Examples.

FIG. **6**A shows the disulfide bonded structure of gp(1-263) V1/V2 fusion glycoprotein described in the Examples.

FIG. **6**B shows an autoradiogram of an SDS PAGE analysis of immunoprecipitated gp(1-263) V1/V2 fusion glycoprotein described in the Examples.

FIG. **7** shows an autoradiogram of an SDS PAGE analysis of immunoprecipitated gp(1-263) V1/V2 fusion glycoprotein described in the Examples.

FIG. **8** shows an autoradiogram of an SDS PAGE analysis of immunoprecipitated gp(1-263) V1/V2 fusion glycoprotein described in the Examples.

FIG. **9** shows an autoradiogram of an SDS PAGE analysis of immunoprecipitated glycosylation site mutant gp(1-263) V1/V2 fusion glycoprotein described in the Examples.

FIG. **10**A shows the disulfide bonded structure of gp(1-263) V3 fusion glycoprotein described in the Examples.

FIG. **10**B shows autoradiogram of an SDS PAGE analysis of immunoprecipitated gp(1-263) V3 fusion glycoprotein described in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

The env-based expression system of this invention allows glycopeptides and viral particles incorporating those glyco-

peptides to be made by mammalian cells in an efficient manner. We have determined that expressed glycopeptides retain glycosylation dependent (such as N-glycan dependent) and conformationally dependent structures. Such expression is particularly important for viral glycopeptides to make them useful for immunoreactive purposes, such as immunoassays or affinity purifications, or for immunogenic purposes, such as the production of monoclonal antibodies or for inducing protective immunity.

It will normally be most desirable to use the invention to express a glycopeptide that corresponds to (i.e., has an amino acid sequence that is the same as, or is derived from, the sequence of) a naturally occurring glycopeptide. We have found that the env expression system of the invention is useful, for example, in efficiently expressing N-glycan dependent epitopes contained in the V2 region of HIV-1 gp120. As described in U.S. application Ser. No. 07/860,889, incorporated by reference herein, the V2 region is recognized by a powerfully neutralizing antibody against an N-glycan dependent epitope. In particular, the antibodies are capable of neutralizing HIV-1 infection at a concentration lower than that of previously described neutralizing antibodies. An example of such an antibody, a monoclonal antibody derived from peripheral mononuclear B-cells of an HIV-immunized chimpanzee, is produced by EBV transformed chimp peripheral blood mononuclear cells deposited at the ATCC, Parklawn Drive, Rockville, Md. on Mar. 10, 1992, and accorded accession no. CRL 10983. New glycopeptides that include the V2 region of gp120 are made according to the invention. They contain an N-glycan-dependent epitope that is reactive with antibodies against N-glycan dependent epitopes of native gp120 but do not contain the immunodominant V3 region. Thus they are especially useful to induce (or measure in an immunoassay) a specific anti-V2 response. Such immunoreactive V2 glycopeptides have not been expressed by others.

The vector of the invention is advantageously used to efficiently express complex conformational epitopes, i.e., those involving several disulfide bonds. For example, we have correctly expressed the V1/V2 region of gp120, perhaps the most complicated portion of that molecule, which in addition to six glycosylation sites, includes six cysteines, all of which are believed to be involved in specific disulfide bonds (Leonard et al., 1990, J. Biol. Chem. 265:10373–10382). The V1/V2 region expressed using the vector has the capability of reacting with antibodies against N-glycosylation dependent epitopes (as described above) and/or with antibodies against conformational epitopes of that region.

This invention allows for the expression of fragments of glycoproteins, or glycopeptides, in such a way as to maximize their synthesis, glycosylation, folding, stability, and secretion. Expression of the fusion glycoprotein on the surface of cells or virus particles is also accomplished. The invention can also provide a "tag" for the detection and purification of glycopeptides that is independent of their own properties. The expression system can also advantageously be used to express glycopeptides in immunogenic form, for example fused to the N-terminal carrier portion of the env surface protein. Thus, the vectors are particularly useful in the expression of glycopeptides containing glycan-dependent or conformationally dependent epitopes, fused to the N-terminal surface protein fragment. If desired, the vector may be used to express a fusion protein as a secreted molecule. Alternatively, sequences can be included in the vector that code for the remaining C-terminal part of the env gene. We have determined that fusion proteins can then be functionally expressed in the envelope of infectious or non-infectious particles of the particular retroviral env used in this invention. In this application, "truncation chimeric (or fusion) glycoprotein" is used to refer to those glycoproteins that are expressed by vectors which do not include sequences coding for the remaining env glycoprotein (in particular the C-terminal globular domain of these env surface protein and the trans-membrane protein). The term "insertion chimeric (or fusion) glycoprotein" is used wherein foreign protein fragments are inserted into the region of the surface protein of these particular env types that links the N-terminal and C-terminal globular domains without loss of sequences of either globular domain, and including the transmembrane protein.

The N-terminal fragment of the surface protein used is a carrier for the glycopeptide or glycoprotein that is to be expressed. Retroviral env is normally expressed as a membrane-associated precursor protein which is processed during transport through the ER (endoplasmic reticulum) and Golgi system by proteolytic cleavage and glycan maturation to form a complex consisting of SU (surface protein), disulfide-linked to the TM (transmembrane) protein. For example, MuLV (surface protein) gp70 is a soluble glycoprotein containing several domains, and is linked to transmembrane protein p15E. (Although the molecular weights vary somewhat, the term "gp70" is used herein to refer to the surface protein of all MuLV virus strains, as well as the highly similar surface protein of the FeLV (Feline Leukemia virus) strains.) The receptor binding domain of gp70 is comprised of the N-terminal region, which is believed to be a structurally independent globular region. This domain contains at least two N-linked glycans. The secondary structure of an ecotropic gp70 has recently been determined, and shows that the twelve cysteines in this region are joined in six internal disulfide bonds. The C-terminal domain of MuLV surface protein is also a globular region that contains 4–5 N-linked glycans and includes the disulfide linkage to p15E.

To make a vector for expressing fusion glycoprotein, a recombinant gene is constructed in which the coding sequence for a selected glycopeptide is fused in frame to the C-terminus of a truncation fragment of the retroviral env surface protein. This fragment codes for the N-terminal domain of the protein that is glycosylated normally and folds into a globular structure. In one embodiment of the invention, the globular structure includes the receptor binding domain of the surface protein, i.e., the domain that binds to its cell surface receptor. In another embodiment of the invention, the globular domain includes the first twelve cysteines of gp70, which are all believed to be involved in intra-domain disulfide bonding.

In a preferred embodiment, the truncation (or insertion) site is selected to be in a region of env surface protein that is believed to be in an extended conformation and to function as a linker between the two globular domains of the surface protein. This region is known to be immunogenic in the FeLV and MuLV viruses. For example, in Friend clone 57 of MuLV, this linker region of gp70 is believed to maximally extend from the Cys-free sequence from residue 185 up to but not including the conserved N-glycan attachment site at residue 302. In other surface proteins, the linker is believed to maximally include the entire cysteine free sequence immediately N-terminal to the conserved N-linked glycosylation site homologous to the conserved N-linked glycosylation site at residue 302 of Friend clone 57 gp70.

In another preferred embodiment of the invention, sequences coding for the C-terminal domain of the surface

protein and for the trans-membrane domain of the env precursor glycoprotein are added at the C-terminus of the fusion protein. These additional sequence can generate membrane bound fusion proteins that are efficiently expressed at the cell surface and on virus particles. The additional sequences can allow expression of the fusion glycoprotein from infectious virus. Coexpression of at least the gag gene from any vector allows incorporation into retroviral particles. This can be advantageous, because, for example, particulate immunogens are believed to generally be more immunogenic than their soluble counterparts.

A particular class of retroviral env proteins that share a specific sequence motif defines those env proteins that are used in the fusion glycoproteins, and coded for by the vectors, of this invention. In particular, the retroviral env proteins are defined by the presence of a hydrophobic glycosylation signal located about seven residues N-terminal to a Cys-trp-leu-cys sequence (SEQ ID NO:18) located centrally in the SU domain of the env polyprotein. This glycosylation signal and sequence have been described by Kayman et al., 1991, J. Virol. 65:5323. These sequences are invariably associated with the presence of a Cys-x-x-x-x-x-x-cys-cys sequence (SEQ ID NO:19) within the ectodomain of the transmembrane protein. Preferred are those env proteins of this type that also display an extensive Cys-free region N-terminal to the hydrophobic glycosylation signal. This cys-free region preferably is at least 40 amino acids long. These include env proteins of the REV-A avian virus (Wilhelmsen, Eggleton and Temin 1984 J. Virol. 52:172–182) and Mason-Pfizer Monkey Virus (Sonigo et al.1986 Cell 45:375–385). More preferred are those in which this Cys-free region is Pro-rich, i.e., contains several Pro residues. This includes the SU of Gibbon Ape Leukemia Virus (Delassus, Sonigo and Wain-Hobson 1989 Virol. 173:205–213). Most preferred for use in this invention are the env genes of MuLV and of Feline Leukemia Virus (e.g. Donahue et al.1988 J. Virol. 62:722–731), which have a very high degree of sequence similarity to each other and also share the above mentioned defining characteristics. These surface proteins have N-terminal and C-terminal globular domains joined by a linker region into which the selected peptide is placed to form a truncation or an insertion glycoprotein.

The sequences encoding the fusion glycoprotein (whether as a secretable separate moiety, an internal protein, a cell surface protein, or associated with retroviral particles) can be expressed using conventional vectors for expression of proteins in mammalian cells. Retroviral expression vectors can be used, such as those developed from Rous sarcoma virus (Sorge et al., 1982, J. Mol. Appl. Gen., 1:547), murine mammary tumor virus (Gunzberg et al., 1986, Virology, 155:236) and murine leukemia virus (MuLV), such as the Moloney MuLV (McLachlin et al., 1990, in Progress in Nucleic Acid Research and Molecular Biology, 38:91) or the Friend MuLV. Secreted fusion glycoproteins or glycoproteins associated with viral particles are produced from a preferred retroviral vector described below. The preferred vector allows easy and rapid manipulation of new constructs, and high level expression.

Alternatively, conventional non-retroviral viral vectors can be conventionally constructed to express the fusion glycoproteins of the invention, including vaccinia (for example, see Vijaya et al., 1988, Vaccines 88, CSH pp.211–214) and herpes virus (for example, see Kit et al., 1991, Vaccine 9:564–572).

In addition to viral vectors, conventional plasmid vector expression systems that do not employ viral particle intermediates can be employed to express the fusion glycoproteins of the invention in mammalian cells. These systems are either transient expression systems (i.e., those that result in death of the mammalian cells producing the expressed product) or those that allow stable producing mammalian cell lines to be established (i.e., do not result in death of the cells).

The systems described above are known in the art and are made using well-established techniques. Conventional cloning vehicles are used to make plasmids encoding the desired vectors using techniques such as described in *Current Protocols in Molecular Biology* (Ausubel et al.eds, John Wiley and Sons, New York, N.Y.) and *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds, Second Edition, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Conventional cell lines, such as mammalian insect, yeast and other glycosylating cell lines, are transformed, transfected, or infected, to generate the fusion glycoproteins of the invention. Cells are also transformed, transfected, or infected in vivo to generate the fusion glycoproteins.

The vectors used in any of these systems for expressing the fusion glycoprotein, i.e. that include a sequence coding for the fusion glycoprotein of the invention operably linked to a promotor, are included in the invention.

The invention also includes the process of infecting mammalian cells with viral vectors, or transfecting the cells with plasmid vectors, of the invention. Mammalian cell lines may be infected when it is desired to purify the fusion glycoprotein for use in an immunoreactive or immunogenic composition. Producer cell lines are made to express secreted fusion glycoprotein or fusion glycoproteins associated with viral particles. It may be preferable to express the fusion glycoprotein in viral particles rather than in soluble form to make recovery easier. Also, infectious or non-infectious particles incorporating the desired glycoprotein in their envelope can be obtained in this manner for immunizing mammals.

Alternately, the vectors can be used to infect or transfect mammalian or other glycosylating cells in vivo in order to cause expression of the soluble glycoprotein, or glycoprotein expressed on the cell surface, or associated with viral particles.

Fusion glycoproteins can be conventionally purified from lysates or supernatants of producing mammalian cell cultures. Soluble glycoproteins are advantageously purified using monoclonal or polyclonal antibodies against the surface protein. If desired, the selected glycopeptide can be cleaved from the env fragment by including a conventional cleavable linker between the N-terminal globular domain and the inserted glycopeptide. If the selected glycopeptide is recovered on a retroviral particle, a cleavable linker can also be included between the selected glycopeptide and the C-terminal env region. The term "fusion glycoprotein," as used herein, refers to products wherein the env sequence is directly fused to the selected glycopeptide as well as wherein there is an additional sequence or sequences in the fusion product. The product may contain the cleavable linker referred to above, and/or other sequences.

For example, a cleavage site for a specific protease could be inserted between the surface protein fragment(s) and the selected glycopeptide in order to allow eventual release of the selected glycopeptide from the env sequence. Incorporation of an Ile-glu-gly-arg sequence (SEQ ID NO:20) allows specific cleavage with blood coagulation factor Xa and has been used successfully to separate (i.e., cleave)

domains of interest from carrier domains in recombinant fusion proteins (Nagai and Thogersen, 1987, Meth. Enzym. 153:461–481).

Also a non-immunological affinity tag can be included at the N-terminus of the surface protein fragment. For example, addition of a sequence of six histidine residues allows rapid purification on an Ni^{2+}-nitrilotriacetic affinity column under native conditions using imidazole buffers (Janknecht et al., 1991, PNAS 88:8972–8976 and Examples below).

T-helper epitopes from the source of the heterologous gene fragment being expressed can also be inserted into the fusion gene at the N-terminus, the C-terminus, or elsewhere, to enhance the immunogenicity of the fusion protein and the probability of a rapid and intense immune response of animals immunized with fusion glycoprotein, following exposure to the pathogen. For purposes of HIV-1 glycopeptide expression, a number of T-helper epitopes with broad MHC reactivity have been characterized, such as amino acids 791–823 from the C-terminus of gp41 or amino acids 391–414 from the C4 region of gp120 (Berzofsky et al, 1991, J. Clin. Invest. 88:876–884).

For expressing certain HIV sequences in fusion glycoproteins of this invention, it may be necessary to include RRE (rev responsive element) sequences in the vector such that they will be retained in the mRNA encoding the fusion glycoprotein and to provide rev activity in cells expressing the fusion glycoprotein. These elements are not required for expressing the V1/V2, V3, or V4/C4 fusion glycoproteins described herein.

It is also possible to express multiple selected glycopeptides in a single fusion glycoprotein. This can be done with either truncation or insertion glycoproteins. For example, the selected glycopeptides can be different fragments of a single protein, fragments from different proteins, or homologous fragments from different alleles of the same protein (e.g. the V1/V2 domain of gp120 from the HXB2d and MN isolates of HIV-1), or any combination of such fragments. The multiple inserts can be placed at different locations within the interdomain linker of an SU. Alternatively, multiple selected glycopeptides can be inserted in concatenated arrays at a single site in the SU. Such inserted multiple glycopeptides can be separated from each other by a spacer peptide.

The N-terminal fragment of the env glycoprotein preferably forms a receptor binding domain, as it is believed that proper autonomous folding and glycosylation of that region during expression may aid independent proper folding and glycosylation of the selected glycopeptide. It may also aid in efficient secretion of the fusion glycoprotein. Examples of such env fragments are amino acids 1–263 and 1–285 of Friend ecotropic clone 57 gp70. An env fragment containing amino acids 1–227 is also described below. These gp70 fragments terminate in or near a proline-rich region that is believed to form an extended linker between N-terminal and C-terminal globular domains of gp70 and is known to be an immunogenic region of gp70. The region carries epitopes recognized by polyclonal hyperimmune sera and monoclonal antibodies. The gp70 domain can therefore serve as a tag for the identification and purification of the fusion glycoprotein. Truncation at amino acid 227 removes the entire proline-rich linker region and some of the previously assigned N-terminal domain but retains all of the N-terminal Cys residues and results in a fragment that possesses receptor-binding activity. Truncation at amino acid 263 retains a large fraction of the proline-rich linker but eliminates the section of this linker region that is known to carry O-linked glycans in gp70 as well as the third N-linked glycan of Friend MuLV gp70. The resultant protein fragment is efficiently expressed and secreted more rapidly than the 227 amino acid fragment. Truncation at amino acid 285 retains the entire proline-rich linker region, including the O-linked glycosylation sites and the additional N-linked glycosylation site, and the resultant fragment is also efficiently expressed and secreted. The optimal truncation site in gp70 may depend on the particular glycopeptide being expressed.

The expression vector of the invention should encode a signal sequence 5' to the surface protein sequence. In the examples below, the native env signal sequence is encoded in the vectors but other signal sequences can be substituted.

It has been determined that if the C-terminal portion of the env gene sequence is also incorporated in the vectors, i.e., where a foreign sequence is inserted into the surface protein between the N-terminal globular domain and the C-terminal domain, many resulting hybrid env proteins are processed, incorporated into cellular membranes and viral particles, and retain the ability to mediate virus infection. Foreign sequences are exposed on intact virions in an area that is highly immunogenic. Inserted sequences can be used to obtain an infectious particle, a non-infectious particle, or an env protein that is processed and expressed at the cell surface, but not incorporated into virus particle.

The processing of a retroviral env product begin with the folding of the glycosylated precursor protein in the endoplasmic reticulum and culminate with the mature SU and TM proteins on the surface of the virus particle. Those proteins mediate binding to the host cell and effect membrane fusion between the viral and host cell membranes, delivering the viral core into the cytoplasm. Insertion chimeric glycoproteins such as those described in the examples below are able to act as SU for infectious virus. Such fully functional chimeric glycoproteins are believed to be more versatile than non-functional glycoproteins. Particular insertion chimeric glycoproteins may be blocked at different stages of this processing or blocked in one or more functions. However, insertion chimeric glycoproteins that do not retain full function are also useful.

An insertion chimeric glycoprotein that is synthesized but that does not fold properly into a compact globular structure with native env disulfide bonds would be expected to be retained in the endoplasmic reticulum until it was degraded intracellularly. Such an insertion chimeric glycoprotein might not be useful for production of purified glycopeptides or as an immunogen for induction of humoral responses, but would likely be most useful for induction of MHC class I-mediated cellular immune responses. For example, it may be desirable to avoid competition between induction of humoral and cellular immune response, as has been suggested for tuberculosis and AIDS. In this case, use of expression vectors encoding insertion chimeric glycoproteins that do not fold and are not transported out of the endoplasmic reticulum would be particularly appropriate. Vectors encoding insertion chimeric glycoproteins that are processed from the endoplasmic reticulum to the Golgi apparatus but do not continue to the cell surface would be useful in the same ways.

Insertion chimeric glycoproteins that are processed to the cell surface are capable of eliciting humoral responses as well as cellular responses. An insertion chimeric glycoprotein that is destabilized in its interaction with the transmembrane protein is rapidly released from the cell surface (i.e. it

is functionally similar to a secreted protein). An insertion chimeric glycoprotein that interacts normally with the transmembrane protein is likely to be present at significant levels on the cell surface. An insertion chimeric glycoprotein that is present on the cell surface would normally be more immunogenic than one that is rapidly released.

Insertion chimeric glycoproteins that are incorporated into viral particles present the selected glycopeptide on a multivalent particulate immunogen. This is a particularly potent method of antigen presentation. It also allows for particle based purification methods for the insertion chimeric glycoprotein and the selected glycopeptide. Such insertion chimeric glycoproteins do not need to be capable of mediating viral infection.

To obtain expression of chimeric glycoproteins that cannot mediate viral infection, pseudotyped retroviral vectors, other viral vectors (see above) or direct DNA transfection can be used. Any insertion chimeric glycoprotein that is processed to the cell surface can be obtained as a purified protein, and used for its desired purpose, e.g., whether used for binding to a receptor, for immunoreacting, or for inducing an immune response. Non-functional insertion chimeric glycoproteins that are incorporated into viral particles can also be used as inactivated particles for the desired purpose.

The fully functional insertion chimeric glycoprotein can be encoded in an infectious retroviral vector, i.e., a retrovirus that causes a spreading infection of retrovirus expressing the insertion glycoprotein. In the appropriate host such a retrovirus establishes viremia in the infected animal, thereby exposing the animal to an increasing dose of the selected glycopeptide of the insertion glycoprotein for an extended time. Infectious retrovirus incorporating the insertion glycoprotein in its envelope can also be expressed in a species that the virus cannot infect. For example, human cells can be transformed or transfected with a vector for expressing infectious ecotropic MuLV that contains the insertion glycoprotein in its envelope. Since ecotropic viruses do not infect humans, this method is equivalent to using a defective virus. In that case, particle associated, cell surface, and soluble forms of the insertion glycoprotein are presented for (if immunogenicity is desired) induction of humoral responses, and intracellular expression for cellular immune response. Alternatively, deletion of substantial parts of the pol gene from the expression vector genome leads to the expression of non-infectious virus particles bearing the insertion glycoprotein, regardless of host. In addition, incorporating the recombinant env gene into the expression vector genome in the absence of the gag gene allows processing to the cell surface, but not particle formation. Also, expressing the surface protein domain of the chimeric env without the transmembrane domain produces a secreted insertion chimeric glycoprotein.

Chimeric surface proteins that function in virus replication can be used to generate hyperimmune sera and MAbs using live virus instead of adjuvants. Chimeric surface proteins that are incorporated into virus particles but are defective for viral replication can also be used. The inoculated animal is exposed to a multivalent, particulate immunogen rather than a soluble protein, which is potent way of presenting many antigens. Preparation of virus for inoculating animals such as mice and rats to prepare MAbs is extremely easy and inexpensive compared with use of purified proteins. Non-infectious, pseudotyped virus particles can be used in any animals, including humans, and are inexpensive and easy to produce. MuLVs, in particular, have a wide host range that allows use of live virus inoculations in a wide range of mammals. Examples described below utilize an ecotropic SU, which allows infection of rats and mice. Use of amphotropic SUs allows retroviral infection of other mammals as well, including humans. Dualtropic and xenotropic SUs allow infection of certain mammals as well. These SUs are well characterized and known to those skilled in the art.

Where fusion glycoproteins are incorporated into viral particles, the size of the particle can be used as a basis for the purification of the fusion glycoprotein and the selected glycopeptide. Because of this particle association, separation from almost all other proteins found in cell supernatants is easily accomplished by ultracentrifugation of the particles.

An inserted protein that is expressed in infectious or non-infectious particles can be used in inactivated particle compositions used, for example, for immunogenic purposes. The infectious or non-infectious particles can also be expressed by inoculating with retrovirus vectors, or with other vectors such as those mentioned above. In the case of immunogenic compositions, preparation of live virus is easier and less expensive than preparation of subunit compositions. Inactivated virus particles are also relatively inexpensive and easy to produce, and often highly immunogenic. It is also possible to purify viral particles of the invention to a higher yield and purity than certain viruses from which the inserted glycoprotein can be derived, e.g., HIV. With respect to HIV, this is due in part to the fact that the surface protein in the present invention is covalently linked to the transmembrane protein whereas in HIV that linkage is non-covalent. Also, nonhazardous particles can be obtained using the invention, whereas HIV is highly pathogenic and therefore difficult to obtain in large quantities. Also, by using multiple allelic antigenic sequences of a virus from which the inserted polypeptide is derived, a broader (i.e., less type-specific) anti-virus response might be induced than by using the virus itself as an immunogen.

DNA compositions such as plasmid DNA vectors can also be used for inoculation. An example of such a vector is described below. Retroviral vectors can also be used in DNA compositions as well as in viral particles.

Expression of the chimeric env gene without the gag gene would produce proteins expressed on the cell surface. The gag gene can be co-expressed with the chimeric env gene in the absence of a functional pol gene to produce defective retroviral particles which present the chimeric retroviral particles on their surface. Such use retains the advantage of cell surface and/or particle presentation of humoral response epitopes and presentation of cellular response epitopes while avoiding potential risks of live retrovirus infection.

The expression vector of the invention can be created from available materials using a shuttle vector for manipulating constructs in, e.g., *E. coli*. Assembly of expression vectors is described in detail in the Examples below. Alternatively, expression vector pLRB332, described in the Examples, can be obtained and is adapted for insertion of a sequence encoding a selected glycopeptide to make an expression vector encoding a fusion product.

Vectors described in the Examples below have a number of advantages:

1) All DNA construction steps can be carried out in bacteria. If desired, resulting plasmids can be used directly to construct mammalian cell lines expressing a recombinant fusion glycoprotein without the need to generate recombinant viruses within infected cells, as is required for other common systems such as vaccinia virus, herpes virus, and baculovirus. This allows large

numbers of constructs to be prepared and analyzed quickly and efficiently.

2) The vector system is non-lytic and thus generates stable cell lines, so that continuously producing cultures can be isolated and used to produce fusion glycoproteins.

3) Cultures in which essentially all cells are expressing the recombinant protein can be prepared quickly, without using selectable markers, by using retroviral vector packaging cell lines.

4) The constructs can be easily expressed in a wide variety of cells, including those of human origin, using amphotropic pseudotypes of the MuLV vector, or by constructing the chimeras in an amphotropic surface protein.

5) The level of expressed protein in mammalian cell culture is quite high, allowing analyses to be carried out easily and quickly during the experimental stages of vaccine development, as shown in the Examples below.

The MuLV vector embodiment of the invention can be manufactured in packaging cell lines such as those that are described in the Examples or any other suitable lines. Such cell lines are well known and available. The producer cell line used, i.e., that which is infected and produces the fusion glycoprotein, can be a conventional mammalian cell line that can be infected with either ecotropic or amphotropic MuLVs. Ecotropic retroviruses infect and replicate only in mouse and rat cells. Amphotropic retroviruses infect mouse as well as other mammalian species. The MuLV virus can also be used to infect mammals to cause in vivo expression of the fusion glycoprotein.

As noted above, the purified products of the expression system can be used as immunogens, either for production of monoclonal or polyclonal antibodies or induction of protective immunity. The products can be mixed with appropriate adjuvants in order to enhance immune response. If the selected glycopeptide is cleaved from the carrier (or the entire envelope, where expressed in particle form), it can be chemically joined to a conventional carrier, such as bovine serum albumen. The fusion glycoprotein, however, when used separately from virus particle as an immunogen, preferably has a molecular weight greater than 20,000 daltons, so that it is likely to be immunogenic by itself Immunogenic compositions can be administered intradermally or subcutaneously or orally. For a vaccine, several inoculations are preferably administered, with follow-up booster administrations.

Conventional assays can be performed with products made using the invention to detect antibodies, receptors, or other binding partners for the expressed glycopeptide. Truncation and insertion chimeric glycoproteins, as well as glycopeptides cleaved from these glycoproteins, are used in these methods. Lower levels of mature insertion chimeric glycoprotein are obtained as compared with truncation chimeric glycoprotein; thus it may be preferable to use the truncation chimeric glycoprotein. A selected insertion chimeric glycoprotein, however, may present epitopes not presented by the corresponding truncation chimeric glycoprotein. The ability to use particle association as the basis for insertion chimeric glycoprotein purification can also make these preferable to truncation glycoproteins. If desired, the insertion chimeric glycoproteins can be separated from the particle and the other viral proteins.

An immunoassay such as an ELISA might employ a soluble fusion product including, for example, the V1/V2 glycopeptide, and also include means for the detection of an immune complex formed between anti-HIV antibody and the fusion product. For example, a "sandwich" might be formed using a solid phase coated with HIV-1 lysate, anti-HIV antibody, and the fusion product, followed by detection of the "sandwich" by a labelled anti-gp70 antibody. Alternatively, a solid phase might be coated with the fusion glycoprotein (or glycopeptide cleaved from the glycoprotein), the solid phase exposed to sera containing anti-HIV antibody, and the presence of the anti-V1/V2 antibody detected with a subsequent labelled anti-human antibody. In another method, radiolabelled fusion glycoprotein is used as a substrate for immunoprecipitation, followed by separation of proteins according to molecular mass on SDS polyacrylamide gels and detection by exposure to photographic film. This is a sensitive method, but it is more labor intensive than ELISA methods. Fusion glycoproteins can also be used in Western Blot methods. Alternatively, a receptor ligand expressed according to the invention can be used in a assay for the receptor. Other diagnostic uses for various expressed glycoproteins made using the invention will be apparent to one skilled in the art.

As noted above, the expression vector is preferably used to express short glycopeptides, for example, less than 150 amino acids, most preferably shorter than 100 amino acids. Often the glycopeptide expressed will be greater than 20 amino acids, embodiments of the examples being greater than 40 amino acids. The invention, however, may also be used for expressing larger fragments or even complete glycoproteins.

Fragments of any of the glycopeptides described herein can also be advantageously expressed as fusion products of the invention.

The invention is further illustrated by the following examples.

EXAMPLES

CONSTRUCTION OF GLYCOPEPTIDE EXPRESSION FUSION VECTORS

Standard recombinant DNA techniques are used throughout, as can be found in manuals such as *Current Protocols in Molecular Biology* (Ausubel et al.eds, John Wiley and Sons, New York, N.Y.) and *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds, Second Edition, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). All relevant work was done using the *E. coli* Strain DH5Alpha (BRL) [Bethesda Research Labs] and the Hanahan (Hanahan, 1983, J. Mol. Biol. 166:557–580) method was used for transformation. Enzymes were from Boehringer-Mannheim and New England Biolabs and were used as recommended by the manufacturers; agarose was from BRL and FMC Bioproducts. All references cited either in the Examples section or in the rest of this application are hereby incorporated by reference.

The MuLV retroviral vector described below has a typical retroviral vector structure, except that gap and pol expression have not been eliminated. Not eliminating gag and pol expression may be desirable for some purposes. The vector uses the natural splice donor and acceptor sequences involved in normal expression of MuLV env in the expression of the fusion glycoprotein.

A one LTR clone of the FB29 isolate of Friend ecotropic MuLV permuted at the unique HindIII site (5060) (Sitbon et al. Cell 47:851, 1986) was used to make a MuLV expression vector. The sequence of this isolate is shown as FIG. **1** (SEQ ID NO:8). Restriction site numbering below refers to the first base of the enzyme recognition site in this FB29 sequence, which begins at the 5' end of the genomic RNA. The entire envelope gene sequence and a portion of the pol gene and the 3' LTR from the clone 57 Friend ecotropic MuLV (Oliff et al. J. Virol. 33:475, 1980; complete sequence

shown in Koch, Nunsmann and Friedrich, 1983, J. Virol. 45:1–9) were substituted for those of the FB29 isolate using the shared, unique restriction sites SphI (5135) and EspI (7863), resulting in an FB29/clone 57 hybrid permuted viral genome. A collinear, two LTR clone was constructed from this isolate as follows.

The unique PvuII site in pSP72 (Promega), a high copy number *E. coli* vector, was converted to an NheI site by insertion of an 8-basepair linker (GGCTAGCC). The EspI (7863) to HindIII (5060) fragment carrying the LTR and the gag gene and part of the pol gene from the FB29 permuted clone was then inserted into HindIII/EcoRV cut plasmid, following *E. coli* DNA polymerase Klenow fragment-filling of the EspI overhang. Then the HindIII (5060) to SpeI (280) (SpeI generates NheI compatible overhangs) fragment from the permuted FB29/clone 57 hybrid clone carrying the rest of the pol gene and the envelope gene and the LTR was inserted into NheI/HindIII cut plasmid. This resulted in a hybrid colinear viral genome, with the 5' LTR beginning at the (destroyed) EspI site and the 3' LTR terminating at the (destroyed) SpeI site, in which all sequences derive from the FB29 clone except those between the SphI and EspI sites. The total plasmid, pLRB303, is 11.32 kb, with unique viral sequences of 8.93 kb (a restriction map of the MuLV sequences is shown in FIG. **2**).

Glycopeptide expression fusion vectors were derived from pLRB303 as follows. Clone 57 sequences containing the SphI site near the 5' end and modified at the 3' end were generated by polymerase chain reaction (PCR). In these modified sequences, the selected fusion sites in the hypervariable region of gp70 are followed by an in-frame NheI site that adds an ala-ser dipeptide to the end of the gp70 fragment, two stop codons (UAA-UGA), and a ClaI site. The 5' primer was 5'-CCAAGAAGCTTCTAGAAGAAA-3' (SEQ ID NO:3), the 3' primer for the gp(1-263) construct was 5'-GGTTATCGATTCATTAGCTAGCGGGGGGA GACTTGGCAGGTT-3' (SEQ ID NO:2) and the 3' primer for the gp(1-285) construct was 5'-CTCAGCCC CCGCCAGCAGGAGCTAGCTAATGAATCGATAACC-3' (SEQ ID NO:1). PCR was carried out using Vent® polymerase (New England Biolabs) in supplied Vent® buffer plus 4 mM $MgSO_4$ and the recommended BSA with 0.5 microM primers, 400 microM each dNTP, 100 ng of pLRB140 (a HindIII (5063) to KpnI (8323) fragment of clone 57 in plasmid pTZ18R (US BIOCHEMICALS) containing the 3' end of the pol gene, all of the env gene, and most of the 3' LTR) in a Perkin Elmer Cetus DNA Thermal Cycler 9810 for 25 cycles of 960 for 1.5 min, 52° for 1.5 min, 72° for 1.5 min. Polymerase was added last to reaction mixtures that were pre-incubated and held at 96°. Following extraction with phenol:chloroform and ethanol precipitation, PCR products were digested with SphI and ClaI and the desired SphI/ClaI fragments purified from agarose gels with Qiaex® Resin (Qiagen) according to the manufacturer's directions. These fragments were then inserted into pLRB303 that had been digested with SphI and ClaI and gel purified. This resulted in the deletion of viral envelope sequences between the desired fusion point and the ClaI (7702) site present near the 3' end of the envelope gene. The gp(1-263) fusion vector was designated pLRB333; the gp(1-285) fusion vector was designated pLRB332. FIG. **3**A shows the complete DNA sequence of pLRB332 (SEQ ID NO:1), and FIG. **3**B shows the amino acid sequence (SEQ ID NO:10–11) of the encoded truncation fragment. FIG. **4**A is a restriction map of the MuLV sequences of pLRB332. The NheI and ClaI sites are used for the insertion of sequences coding for the glycopeptide that is to be expressed. The structures of these plasmids were confirmed using NdeI, BamHI, SphI/NheI, SphI/ClaI, and NheI/ClaI restriction digests.

pLRB332 (SEQ ID NO:9) was deposited at the ATCC, Parklawn Drive, Rockville, Md. on Aug. 25, 1992 and assigned accession no. 69057. pLRB332 can be converted to the sequence of pLRB333 (i.e., encoding gp(1-263)) by taking a PCR-generated SphI/ClaI fragment made from pLRB332 (SEQ ID NO:9) using the primers described for construction of pLRB333 and substituting this fragment for the SphI/ClaI fragment carried by pLRB332 (SEQ ID NO:9).

CONSTRUCTION OF GENES EXPRESSING TRUNCATION FUSION GLYCOPROTEINS

For insertion of gene fragments into pLRB332 (SEQ ID NO:9) or pLRB333, PCR is used to generate DNA fragments containing an in-frame restriction site for NheI (results in an ala-ser linker between the gp70 fragment and the inserted glycopeptide) 5' to the sequence encoding the glycopeptide to be expressed and following this sequence two in-frame stop codons followed by a ClaI restriction site. If the desired gene fragment contains an NheI site, an AvrII site (results in an arg-arg linker) or SpeI site (results in an thr-ser linker) or XbaI site (results in an ser-arg linker), each of which result in NheI compatible ends, can be used instead; if the desired gene fragment contains a ClaI site, a BstBI or AccI site could be substituted. These restriction sites are used to insert the gene fragment into the expression vector, generating a gene that can express the fusion glycoprotein. A NarI or other appropriate restriction site (i.e., one not present in pLRB332 (SEQ ID NO:9) or pLRB333 or the gene fragment to be expressed and, if possible, coding for amino acids such as ala, gly, pro, ser, or thr, that are unlikely to constrain the conformation taken by the fusion glycoproteins) can be included between the gene fragment and the stop codons to allow the subsequent insertion of additional sequences at the 3' end of the fusion protein.

If all of the restriction enzymes having either NheI or ClaI compatible overhangs have at least one recognition site in the desired sequence, a new fusion vector can be generated using restriction sites for insertion that are not present in the gene fragment of interest. One can also mutate undesired restriction sites in the sequences coding for selected glycopeptides to facilitate construction. Alternatively, one can use DNA fragments from partial digests for constructions.

For construction of genes to express fusion glycoproteins containing the V1/V2 domain of HIV-1 HXB2d (amino acids 86–179 of the mature gp120) NheI and ClaI sites were used. The 5' primer was 5'-CATCGCTA GCCTAAAGCCATGTGTAAAATTA-3' (SEQ ID NO:4) and the 3' primer was 5'-ACTGATCGATTCATT AGGATACCTTTGGACAGGCC-3' (SEQ ID NO:5). The DNA substrate was 100 ng of HXB2-env (Page et al, 1990, J. Virol. 64:5270–5276) but any other source of HXB2d envelope sequences is equivalent. PCR conditions were as described above for generation of pLRB332 (SEQ ID NO:9) and pLRB333 except for using dNTPs at 200 microM. NheI/ClaI digested PCR-generated fragments were gel purified and ligated to pLRB332 (SEQ ID NO:9) and pLRB333 that had been NheI/ClaI digested and gel purified, generating pLRB335 and pLRB336, respectively. The structures of these plasmids were confirmed with NdeI, NheI/ClaI, and NsiI/NdeI restriction digests.

For construction of genes to express fusion glycoproteins containing the V3 domain of HIV-1 HXB2d (amino acids 261–306) NheI and ClaI sites were used and a NarI site was included between the HIV sequences and the stop codon,

adding gly-ala to the C-terminus of the fusion protein. The 5' primer was 5'-CGGTGCTAGCTCTGTAGAAATTAA TTGT-3' (SEQ ID NO:6) and the 3' primer was 5'-CTAGATCGATCTATTAGGCGCCTGCTCTACTAAT GTTACA-3' (SEQ ID NO:7). Other components and conditions were as described above for the V1/V2 constructions except that $MgSO_4$ was not added. Insertion of PCR-generated fragments into expression vectors was as described for V1/V2 constructions. The gp(1-263)-V3 construct was designated pLRB350 (this construct has also been referred to as pLRB346). The gp(1-285)-V3 construct was designated pLRB349. Plasmid structures were characterized with NdeI, AseI, and NheI/XbaI restriction digests. The ClaI sites were not characterized because in these constructs there is C residue 3' to the ClaI site, resulting in Dam methylation that blocks cleavage by ClaI.

The produced truncation fusion glycoprotein is diagrammed in FIG. **4**B.

CONSTRUCTION OF GENES EXPRESSING INSERTION FUSION GLYCOPROTEINS

Two sites within the inter-domain spacer sequence of gp70 were used above as fusion points for soluble chimeric proteins (i.e., insertion sites for heterologous protein fragments). pLRB349 and pLRB350 contain genes for truncation chimeric glycoproteins with insertions of the HXB2 V3 loop sequence following residues 285 and 263 respectively. The insertions were made to include a NarI site adjacent to and in frame with the C-terminal sequence of the truncation fusion proteins. This unique NarI site was then used to replace the missing env gene sequences between the truncation point and the ClaI site near the 3' end of the env gene. The necessary env gene sequences beginning with a NarI site were generated by PCR from pLRB303. The 5' primer for the fragment beginning with residue 263 was 5'-TCCT GGC GCC TCT AAT TCG ACT CCC ACA TT-3' (SEQ ID NO:12) ; the 5' primer for the fragment beginning with residue 286 was 5'-TTGC GGC GCC ACG GGA GAC AGG TTA CTA AAT C-3' (SEQ ID NO:13) ; the 3' primer for both fragments was 5'-ACTG TCT AGA AAG CGC GCG AAC AGA AGC GAG AAG C-3' (SEQ ID NO:17). Genes for truncation chimeric fusion glycoproteins containing a selected glycopeptide other than V3 can be converted to insertion chimeras (containing the complete env protein) by the same process used to generate the V3 insertion chimeras provided they contain a NarI or other suitable site at the 3' end of the inserted gene fragment. Alternatively, other heterologous sequences can be incorporated into the V3 insertion chimeras directly by removal of the V3 gene fragment using the NheI and NarI sites and replacing it with the appropriate NheI-NarI bounded gene fragment generated by PCR or otherwise. Using these methods with NheI and NarI sites, the complete insert consists of Ala-Ser-Heterologous Sequence-Gly-Ala (SEQ ID NO:21).

To generate an insertion chimeric glycoprotein virus plasmid one can also begin with pLRB332 (SEQ ID NO:9). If a 263/264 insertion is desired, pLRB332 is first modified as described above for the generation of pLRB333. Next, a truncation chimeric glycoprotein plasmid is constructed that incorporates a NarI site at the 3' end of the inserted gene fragment, as described above for the construction of the V3 truncation chimeric glycoprotein genes in pLRB349 and pLRB350. Finally, the truncation chimeric glycoprotein gene is converted to an insertion chimeric glycoprotein gene as described in the preceding paragraph. Using this method, any source of the clone 57 env gene that includes the 3' LTR, in which the designated 3' primer is located, can serve as substrate. A shorter clone that includes the necessary sequences of the env gene, e.g. without unnecessary LTR sequences, can also be used as a source of the C-terminal env sequences by using a different 3' primer.

EXPRESSION OF TRUNCATION FUSION GLYCOPROTEIN VECTORS

Standard cell culture and retrovirological methods were used (see, e.g. Kayman et al, 1991, J. Virol. 65:5323 and references cited therein for general methods and co-culture methods for expression of defective viral genomes).

Column-purified, or boiling mini-prep, glycopeptide fusion vector plasmid DNA (Qiagen or Nest Group kits) is transfected into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986, Mol. Cell. Biol. 6:2895) by conventional calcium phosphate methods using CellPhect reagents (Pharmacia) (or by Lipofectamine (BRL) transfection according to manufacturer's specifications) and then cells of the ecotropic packaging line psi2 (Mann et al, 1983, Cell 33:153) were added. Amphotropic pseudotypes of the glycopeptide fusion vector from PA317 cells infect psi2 cells and ecotropic pseudotypes from psi2 cells infect PA317 cells, allowing the fusion vector to spread through the culture, resulting in high level expression of the fusion protein in the culture, as well as production of high titers of pseudotyped fusion vector virus particles, usually within three-four days of establishing the co-culture. These co-cultures of PA317 and psi2 cells expressing the fusion glycoprotein can be used directly to characterize the biochemical and immunoreactive properties of the fusion protein. To eliminate the presence of helper virus envelope proteins, the pseudotyped vector virus from these cultures was used to infect NIH 3T3 cells and clonal infected cell lines generated by limiting dilution.

The produced insertion fusion glycoprotein is diagrammed in FIG. **4**B.

EXPRESSION OF INSERTION FUSION GLYCOPROTEIN VECTORS

Functional insertion chimeric glycoproteins are expressed by transfection into 3T3 cells. For preparing pseudotypes of infectious chimeras, virus from these cells can be infected into appropriate packaging cell lines. For example, a virus coding for an ecotropic MuLV can be used to infect an amphotropic packaging cell line, such as the PA317 line. This produces pseudotyped virus that will infect, e.g. humans, but will not cause a spreading viremia, except in mice and rats. For non-functional insertion chimeric glycoproteins or for functional insertion chimeric glycoproteins expressed from retroviral vectors without either a functional gag or pol genes, expression is carried out in packaging cocultures as described for truncation chimeric glycoproteins.

GENERAL METHODS FOR ANALYSIS

Characterization of the fusion proteins was done by radioimmunoprecipitation assay (RIPA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), methods for which are referenced in Kayman et al, 1991, J. Virol. 65:5323, and by ELISA as described herein.

V1/V2 domain synthetic peptides ADP 740.9 through ADP 740.17, which are each 20 amino acids long and offset by 10 amino acids and match HXB2 gp120 sequences from amino acids 82 through 181, were obtained. Additional V2 domain 16 mer oligopeptides matching amino acids 135–149 of the HXB2d sequence (ADP 794.2) and the homologous sequences from MN (ADP 794.3) and RF (794.4), each containing an additional C-terminal cys residue, were also obtained. The analogous peptide matching the consensus sequence for this region as defined in the Los Alamos Human Retroviruses and AIDS database, Ile-

Arg-Asp-Lys-Val-Gln-Lys-Glu-Tyr-Ala-Leu-Phe-Tyr-Lys-Leu-(Cys) (ADP 794.1), (SEQ ID NO:22) was also used. A linear V3 peptide corresponding to the complete sequence between the Cys residues defining the V3 loop of HXB2, Thr-Arg-Pro-Asn-Asn-Asn-Asn-Thr-Arg-Lys-Ser-Ile-Arg-Ile-Gln-Arg--Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala-His, (SEQ ID NO:23) was obtained. Peptide ADP 792.3 had the same sequence for the V3 loop and included the defining Cys residues and a C-terminal Asn. It was obtained as the "cyclic" form. An MN V3 peptide, ADP 715, Arg-Lys-Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Thr-Lys-Asn (SEQ ID NO:24), corresponded to the tip of the V3 loop.

To facilitate purification of the gp(1-263):$V3_{HXB2}$ fusion glycoprotein, His^8Gln^9 of gp70 were replaced with a sequence of six His residues. PCR overlap mutagenesis (Ho et al., 1989, Gene 77:51–599) was used to construct the appropriate SphI to NheI gene fragment for insertion into the expression vector. Supernatants from 3T3 cells expressing the His_6 form of gp(1-263):$V3_{HXB2}$ were dialyzed against PBS (pH 8), NaCl was added to 0.5M, and protein was bound to Ni^{2+}-nitrilotriacetate Sepharose (Qiagen) in this buffer. gp(1-263):$V3_{HXB2}$ was eluted with 30 mM imidazole in PBS (pH 7.4) following a 20 mM imidazole wash, and constituted only a small fraction of the Coomassie-staining material in these preparations. Comparison with bovine serum albumin standards yielded an estimate of 3 μg of partially purified gp(1-263) :$V3_{HXB2}$ isolated per ml of culture supernatant.

ELISAs were performed in TiterTek Immuno-assay plates (Flow Laboratories). Antigens were adsorbed to wells for 60 min in 100 μl carbonate buffer (pH9.6) washed with PBS/0.05% Tween, blocked for 90 min with 2% BSA in PBS, and washed again with PBS/0.05% Tween. 100 μl of serum diluted in PBS was added for 60 min at RT, and wells were washed with PBS/0.05% Tween, incubated for 60 min with 100 μl alkaline phosphatase coupled goat anti-human IgG (Zymed) diluted in 2% BSA, washed in PBS/0.05% Tween, and 100 μl of 1 mg/ml p-nitrophenol phosphate in diethanolamine buffer (pH 9.8) was added. Absorbance at 405 nm was measured between 30 and 60 min after substrate addition. The amount of partially purified gp(1-263):$V3_{HXB2}$ used per assay was always sufficient to give at least 75% of the maximum achievable signal. Peptides were used at 100 ng per well; assays were insensitive to increased amounts of peptide. Background A_{405} reaction in wells lacking antigen was subtracted from the data obtained.

ANALYSIS OF FUSION PRODUCTS

a. Analysis of synthesis and secretion of the gp(1-263) and gp(1-285) truncated products

3T3 cells expressing either gp(1-263) (top) or gp(1-285) (bottom) were pulse labeled with 35 ^{35}S-cysteine for 30 minutes (lanes p) and chased with unlabeled medium for 1, 2, 4 or 6 hrs. Cell lysates and supernatants were then immunoprecipitated with a polyclonal goat anti-gp70 serum (goat anti-Rauscher gp70 , Microbiological Associates), and analyzed by SDS-PAGE. Results are shown in FIG. **5**.

Results

This experiment documents the efficient synthesis and secretion of both the gp(1-263) and gp(1-285) truncated gp70 products. For the gp(1-263) construct, at the end of the 30 minute pulse a single band of about 38 kD was seen in the cell extract. After the 1 hr chase, greater than 90% of this material was found in the supernatant medium with less than 10% left in the cells. After 2 hrs of chase, a similar level of protein was detected in the medium, while at longer periods the amount recovered starts decreasing, presumably indicating degradation.

Similar results were found for the gp(1-285) construct. In this case two bands were seen in the cell extracts, a major 43 kD band representing the precursor form, and a 48 kD band representing O-glycosylated product. After a 1 hr chase period, almost all of the labeled material had been secreted into the extracellular medium in the form of a major 48 kD band and a minor 50 kD band. The small amount of material left in the cells seems stable, and presumably represents a fraction of misfolded protein which cannot be fully processed and secreted.

b. Analysis of immunoreactivity of gp(1-263)-V1/V2 fusion product.

FIG. **6**A shows the structure of the fusion protein, showing disulfide bonds and glycosylation sites of both the gp70-derived region and the gp120-derived region. The two regions are separated by an ala-ser dipeptide linker marked by a triangle. The two N-linked glycosylation sites in the gp70 region are residues 12 and 168 and are indicated by circled numbers. The glycosylation sites in the V1/V2 domains are indicated by the branched structures.

FIG. **6**B shown an analysis of the immunoreactivity of the secreted gp(1-263)-V1/V2 fusion protein. Packaging cell cultures expressing the gp(1-263)-V1/V2 product were labeled with 100 uCi of 35^{35}S-cysteine overnight. Cell supernatants were immunoprecipitated with MAbs CRA-3 (lane **1**), C810G (lane **2**), G3–4 (lane **3**), sera from chimp 087 (lane **4**), anti-gp70 MAb 273 (lane **5**), and goat anti-gp70 serum (lane **6**). Preimmune chimp and goat sera were negative, as were a number of other monoclonals against different sites on gp120. The polyclonal anti-gp70 serum also precipitates a MuLV gp70 band that is derived from the helper virus in the packaging cell line.

Results

The structure of the gp(1-263)-V1/V2 fusion product is indicated in FIG. **6**A. FIG. **6**B shows that the expressed protein is recognized by anti V1/V2 MAbs that are dependent on glycosylation (CRA3, C108G and G3–4) and conformation (G3–4 and CRA3). This shows that proteins are expressed that are correctly folded and glycosylated.

c. Analysis of sera from HIV-1-seropositive hemophiliacs for antibodies reactive with the HXB2 V1/V2 fusion protein gp(1-263)-V1/V2.

3T3 cultures expressing the gp(1-263)-V1/V2 fusion product were radiolabeled with 100 uCi of ^{35}S-cysteine overnight, and supernatant medium was immunoprecipitated with a group of sera of HIV-1-infected hemophiliacs (lanes **1–11**). Radioimmunoprecipitations were performed as described for FIG. **6**. All sera were tested at a dilution of 1:50. Results are shown in FIG. **7**.

Results

A reasonable percentage of HIV seropositive human sera contains low titers of antibodies that recognize the gp(1-263)-V1/V2 protein (lanes **1,3,5,9,11**), and one patient serum (lane **4**) possessed particularly potent precipitating activity against this construct. This suggests that most humans are capable of producing antibodies against the V1/V2 region. This result further suggests that the HXB2 V1/V2 sequence is either a fairly common component in the panoply of viruses seen by these patients, or it contains epitopes that are crossreactive with those seen in the V1/V2 domains of the viruses infecting these patients.

d. Quantitative immunoprecipitation of gp(1-263)-V1/V2 by goat anti-Rauscher gp70 serum.

Supernatant medium containing radiolabeled gp(1-263)-V1/V2 protein was immunoprecipitated sequentially 3 times with a 1:40 dilution of goat anti-gp70 serum (lanes **1–3**) and then with a 1:100 dilution of serum from a chimp with a high

titer of anti-V/V2 antibody (lane **4**). Results are shown in FIG. **8**. Immunoprecipitation by the chimp serum without preclearing with the goat anti-gp70 serum is shown in lane **5**.

Results

The complete removal of chimp serum immunoprecipitable material by the goat anti-gp70 serum (lane **4**) shows that the goat anti-gp70 serum can quantitatively immunoprecipitate all of the gp(1-263)-V1/V2, including the fraction recognized by the chimp anti-V1/V2 antibodies. Goat serum can therefore be used to purify the fusion proteins by immunoaffinity methods.

e. Analysis of immunoreactivity of glycosylation site mutants of gp(1-263)-V1/V2 (FIG. **9**).

Medium containing radiolabeled fusion proteins from cells expressing wild type gp(1-263)-V1/V2 (WT) and gp(1-263)-V1/V2 in which either the asn at position 186 (186^{-}) or the one at 197 (197^{-}) mutated to gln was immunoprecipitated with either goat anti-gp70 serum (lane A), MAb C108G (lane B), or MAb CRA3 (lane C). The wild type protein runs at a position corresponding to a molecular weight of 65 kD, while the mutant proteins are about 2 kD smaller. The two mutant proteins were grown in the packaging cell cultures, which contain a gp70 band contributed by the packaging virus, that is recognized by the goat anti-gp70 serum.

Results

The wild type gp(1-263)-V1/V2 and the two glycosylation mutants are recognized equally well by the goat anti-gp70 serum, but the mutants are recognized only poorly, if at all, by the two monoclonal antibodies. The 197 mutant was not recognized by CRA3 at all, while both mutants react only weakly with C108G. These results document the fact that both the 186 and 197 sites are glycosylated in the wild type fusion protein, and that both the CRA3 and C108G epitopes are dependent on N-linked glycans at these two positions for proper expression.

f. Structure, expression and immunogenicity of the gp(1-263)-V3 fusion protein (FIG. **10**).

In FIG. **10**A the sequence of the gp(1-263)-V3 fusion protein is shown, showing the gp70 domain, the ala-ser linker dipeptide (in triangle) and the fragment of HXB2 gp120 containing amino acids 261–306. This region includes the Cys_{266}–Cys_{301} disulfide bond, the conserved N-linked glycosylation site inside the V3 loop at position 271, and the two conserved N-linked glycosylation sites immediately outside the V3 loop at positions 265 and 302. FIG. **10**B shows an analysis of the structure and immunoreactivity of the expressed fusion product. Cells expressing the V3 fusion protein were labeled with ^{35}S-cysteine and supernatant medium immunoprecipitated with sera obtained at different dates from an HIV^{III}-infected chimp (lanes **1–7**) or with goat anti-gp70 serum (lane **8**). A fusion product of ~52 kD was recognized by the chimp and goat sera but not by sera from normal chimp or goat sera (not shown).

Results

These experiments demonstrate the efficient synthesis and secretion of the gp(1-263)-V3 fusion construct in immunologically active form. The size of the expressed product suggests that the three glycosylation sites included in the V3 sequences are all utilized.

g. V3 chimeras

pLRB386 carries the HXB2 V3 domain at the 263/264 site on the 46 amino acid insert that contains 3 N-linked glycans and forms an 36 amino acid disulfide-linked loop. The recombinant env for the insertion chimera was produced in an otherwise wild type MuLV genome. When pLRB386 was transfected into 3T3 cells a spreading viral infection resulted. The growth rate of the recombinant virus was similar to that of wild type virus. By immunofluorescence, the recombinant gp70 in the intact virion was shown to present HIV-I epitopes seen by human and chimpanzee type-specific sera and by a potent neutralizing monoclonal antibody, 41.1, that is specific for an epitope in V3 not presented by synthetic peptides. Anti-V3 sera and MAbs were found to also immunoprecipitate intact virions containing the hybrid protein, indicating that these epitopes are highly exposed on the surface of intact virions. Thus, the V3 chimeric virus retains normal infectivity, and expresses a conformational epitope in V3 (the epitope for MAb 41.1) that is a potent target for neutralizing antibodies.

The amount of mature V3 chimeric gp70 produced was similar to the amount of wild type gp70 produced. A large excess of secreted N-terminal protein fragments was cleaved at a site believed to be within the V3 loop from the precursor chimeric gPr80 present in the endoplasmic reticulum. The C-terminal fragment of the cleaved precursor was degraded intracellularly. A percentage of wild type gPr80 was also degraded intracellularly without secretion of detectable fragments.

This V3 insertion chimeric virus appeared to be infectious and immunogenic in rats. Following subcutaneous injection into rats, ELISA titers vs. gp160 were detectable within 4 weeks and rose continuously for at least 18 weeks. Such an extended response to a single exposure strongly suggests that viremia was established in these animals.

A series of V3 insertion chimeras was produced and partially characterized. These included insertion glycoproteins in which the HXB2 V3 peptide was inserted at the 263/264 (pLRB386) and 285/286 sites (pLRB396), between aa 263 and 286 (pLRB395) (i.e., deleting residues 264 through 285, really 265 through 285 since aa 264 is fortuitously restored by the NheI site), and between aa 285 and 264 (pLRB392) (i.e., between repeated sequences of aa 264–285). Also, a mutant sequence in which the Cys residues defining the V3 loop were changed to Ser residues was inserted at the 263/264 site (pLRB393). These constructs had close to normal or normal growth characteristics in tissue culture and expressed the native epitope for MAb 41.1. An infectious insertion chimera carrying the MN-like V3 domain from the Jr-CSF isolate inserted at 263/264 (pLRB410) of HIV-1 was also produced. All of the V3 insertion chimeras generate high levels of a proteolytic fragment cleaved within the V3 loop, as described above.

h. V1/V2 chimeras

The HXB2 V1/V2 domain is contained within the 94 amino acid fragment (amino acids 86–179 of mature gp120) that includes three disulfide bonds. Two of these disulfide bonds generate the V1 and V2 variable loops separated by a short stretch of conserved sequence and the third disulfide bond generates an arm of conserved flanking sequences. The expressed sequence includes six signals for N-linked glycosylation, all of which have been reported to be utilized and one of which (attached to Asn^{156}) was found to be necessary for viral growth in cell culture. The 5' primer for producing the V1/V2 fragment was the same as used to produce the fragment for the truncation chimeric glycoprotein. The 3' primer was 5'-ACTG ATC GAT TCA TTA GGC GCC GGA TAC CTT TGG ACA GGC C-3' (SEQ ID NO:14), which incorporated the NarI site needed for the insertion chimeric glycoprotein that was absent from the 3' primer used to generate the V1/V2 fragment for the truncation chimeric glycoprotein. This gp120 fragment was inserted into the 263/264 site by replacement of the NheI/

NarI V3 gene fragment of pLRB386 to produce pLRB401. This vector expresses a viable MuLV with a gp70 of appropriate size that is recognized by type specific chimpanzee sera, by rat anti-V2 MAb 10/76b, and by a human serum that has cross-reactive anti-V1/V2 antibodies.

i. V4/C4 chimeras

The V4/C4 domain of HXB2d is contained within an 80 amino acid fragment (residues 342–421) that includes two disulfide bonds, one of which generates the V4 loop, and both of which are involved in forming the majority of the C4 region into a loop. 21 amino acids of the C3 region are included in this construct, and the last 12 amino acids of the C4 region are not. The sequence includes 5 signals for N-linked glycosylation, all of which have been reported to be utilized and none of which was found to be necessary for viral growth in cell culture. The 5' primer for producing the V4/C4 fragment was 5'-CATC GCT AGC GTA ACG CAC AGT TTT AAT TGT GGA-3' (SEQ ID NO:15). The 3' primer was 5'-ACTG ATC GAT CTA TTA GGC GCC CCC TGT AAT ATT TGA ACA T-3' (SEQ ID NO:16). The vector expressing this insertion chimera, pLRB408, was constructed in the same manner as pLRB401. It expresses infectious MuLV with a gp70 of appropriate size that is recognized by rat anti-C4 MAb 38.1

The interdomain linker region of gp70 thus appears to be remarkably tolerant of both insertions and rearrangements.

USE OF CHIMERIC GLYCOPROTEINS TO ANALYZE IMMUNE SERA

A fusion glycoprotein containing the V3 domain of the IIIB strain of HIV-1, gp(1-263):$V3_{HXB2}$, was recognized by sera from a human and a chimpanzee that had been infected by HIV_{IIIB} but not by sera from hemophiliac patients that had been infected with HIV-1 viruses of MN-like V3 serotype. The reactive sera had approximately five-fold higher ELISA titers when assayed on gp(1-263):$V3_{HXB2}$ than on matching V3 peptides. Immunoprecipitation of this fusion glycoprotein by the human serum was only partially blocked by V3 peptide, demonstrating that this infected individual produced antibodies against epitopes in V3 that were expressed on the fusion glycoprotein but not by synthetic peptides. A fusion protein containing the HXB2 V1/V2 domain was recognized by the HIV_{IIIB}-infected patient serum as well as by 17 out of 36 HIV-1 seropositive hemophiliac, gay male and intravenous drug user patient sera. Many of these HIV^{+}human sera reacted with V1/V2 domains from several HIV-1 clones expressed in fusion glycoproteins, (Jr-CSF, pLRB357; NL4-3, pLRB359; SF2, pLRB360; MN-ST, pLRB361; Jr-FL, pLRB362). These results indicate the presence of cross-reactive antibodies against epitopes in the V1/V2 domain. Recognition of gp(1-263):$V1/V2_{HXB2}$ by the HIV_{IIIB}-infected human patient serum was largely blocked by synthetic peptides matching V1 but not V2 sequences, while recognition of this construct by a broadly cross-reactive hemophiliac patient serum was not blocked by individual V1 or V2 peptides or by mixtures of these peptides. These data demonstrated that the chimeric glycoproteins described here effectively present native epitopes present in the V1/V2 and V3 domains of gp120 and provide efficient methods for detection of antibodies directed against native epitopes in these regions.

EXPRESSION OF FUSION GLYCOPROTEINS USING VACCINIA

One can express a fusion glycoprotein of this invention from a vaccinia virus vector. One such vector is the recently developed NYVAC vector, a highly attenuated strain of vaccinia virus that is able to elicit immune responses to foreign proteins inserted into the viral genome (Tartaglia et al., 1992, Virology 188:217–232). Although unable to replicate on human derived cells, NYVAC does infect various human cells and allows for the expression of foreign proteins in human cells. Virus can be grown, amplified, and manipulated in Vero cells (ATCC No. CCL81) or primary chick embryo fibroblasts. To insert a foreign gene into a vaccinia virus, an intermediate plasmid vector is constructed in which the selected gene is appropriately linked to the thymidine kinase gene (tk) promoter. Plasmids carrying a fragment of the vaccinia genome surrounding the tk gene in which tk gene sequences need to be replaced with a multiple cloning site, such as pSD460 (Tartaglia et al., 1992, Virology 188:217–232), are used for this purpose. Standard recombinant DNA techniques are used to insert the selected fusion glycoprotein gene into such a vector. If an insertion chimeric glycoprotein is to be expressed along with a gag gene to provide for defective particle formation, it is also incorporated into pSD460 or its equivalent between the flanking vaccinia sequences. It also needs to be associated with a promoter, which can be a second copy of the vaccinia tk promoter. Alternatively it can be associated with a heterologous promoter such as the enhancer/promoter sequences from the widely used immediate early gene of human cytomegalovirus. Following construction of the plasmid vector containing the selected gene or genes associated with promoters (and situated between flanking vaccinia sequences from the tk region of the virus genome), in vivo recombination is used to introduce the selected genes into the complete virus genome. This is accomplished by co-transfecting the plasmid vector DNA and NYVAC genomic DNA into a cell line, such as Vero, to allow recombinants to form. Recombinant virus is identified by plaque hybridization with radiolabelled DNA probes for the inserted genes. The recombinant virus is used for inoculation of mammals or infection of cells in culture for production of fusion glycoproteins.

PLASMID VECTORS FOR EXPRESSION OF FUSION GLYCOPROTEINS

One can express a fusion glycoprotein of the invention from a non-replicating vector such as pRc/CMV (Invitrogen, San Diego Calif.). Standard molecular biological techniques are used to insert the gene for the selected env fusion glycoprotein into the multiple cloning site adjacent to the enhancer/promoter sequences from the immediate early gene of the human cytomegalovirus. If it is desired to express a gag gene to allow particle formation, the gag gene could be substituted for the neomycin gene in this vector that is expressed from a Rous sarcoma virus LTR. Following insertion of the selected gene or genes into the plasmid vector, the plasmid DNA is transfected into appropriate mammalian cells, such as mouse 3T3 or Vero monkey, for expression of fusion glycoprotein in culture. If the neomycin gene of the vector has not been removed, transfectant lines are selected for G418 resistance and screened for appropriate production of the fusion glycoprotein. If the neomycin gene has been removed, the plasmid vector for expression of the fusion glycoprotein is co-transfected with 1/25 as much of another vector that carries a drug-selectable marker to allow selection of transfectant cell lines. To use a plasmid vector for expression of fusion glycoproteins in mammals, direct DNA immunization is used. Purified plasmid DNA is inoculated into the mammal by any of a number of methods, for example, by intramuscular injection of non-replicating expression vector DNA. This method has been shown to be effective for immunizing mice with influenza A nucleoprotein (Ulmer et al 1993 Science 259:1745–1749).

PARTICLE BASED PURIFICATION

This invention can be used as a method for the production and purification of specific glycopeptides. The amount of insertion chimeric glycoprotein produced is generally less than the amount of the corresponding truncation chimeric glycoprotein. This is due to the relatively inefficient processing of the precursor protein encoded by complete env genes with or without insertions. The lower amount produced, however, may be balanced by the fact that the insertion chimeric glycoproteins will, in general, be associated with virus particles rather than as soluble proteins like the truncation chimeric glycoproteins. The virus particles and associated insertion chimeric glycoproteins are separated from soluble proteins by, e.g., size exclusion chromatography (Pinter, Honnen and Tilley 1993 J. Virol. In Press) or filtration through substrates with appropriate pore sizes, as well as by sedimentation velocity or sedimentation density methods, methods that are used on preparative scales. Following separation from soluble components, the insertion chimeric glycoproteins are released from the particle by conventional methods such as: reduction of disulfide bonds; treatment with detergents such as octylglucoside, NP40, or Triton X-100; treatment with chaotropic agents such as guanidine hydrochloride, urea, or lithium chloride. Treatments that have a negative impact on the properties of the glycopeptide being produced are avoided. Following release of the insertion chimeric glycoproteins, the glycoproteins are purified away from the other viral proteins. If the viral membrane has not been disrupted, the methods used to separate the particles from soluble proteins can be used to separate the fusion glycoproteins from the other particle associated proteins. If the viral membrane has been solubilized but the viral core remains intact, the insertion chimeric glycoproteins can be separated from the core proteins as above, and the insertion chimeric glycoproteins purified away from the transmembrane viral protein by standard protein chemistry techniques. If the entire viral particle is disrupted and its component proteins solubilized, the insertion chimeric glycoproteins can be purified from the other viral protein by standard protein chemistry techniques. This is easier than purifying a soluble protein from cell supernatants. The fraction of the mixture that is fusion glycoprotein is greater and there are fewer contaminating proteins.

Following purification of the insertion chimeric glycoproteins, the selected glycopeptide can be cleaved from the carrier SU protein domains. To do so, cleavage sites for sequence specific proteases are incorporated into the insertion chimeric glycoproteins both immediately N-terminal and immediately C-terminal to the glycopeptide. Different cleavage targets can be used on either side of the glycopeptide. One site can be the blood coagulation factor Xa cleavage site (see above), and the other can be the Leu-val-pro-arg-gly-ser (SEQ ID NO:25) cleavage site of thrombin. The selected glycopeptide is then purified from the fragments of SU (and the proteases, unless immobilized proteases are used) by standard protein chemistry techniques.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 25

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCAGCCCCC GCCAGCAGGA GCTAGCTAAT GAATCGATAA CC 42

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTTATCGAT TCATTAGCTA GCGGGGGGAG ACTTGGCAGG TT 42

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCAAGAAGCT TCTAGAAGAA A 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATCGCTAGC CTAAAGCCAT GTGTAAAATT A 31

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACTGATCGAT TCATTAGGAT ACCTTTGGAC AGGCC 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGGTGCTAGC TCTGTAGAAA TTAATTGT 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTAGATCGAT CTATTAGGCG CCTGCTCTAC TAATGTTACA 40

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8323 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGCCAGTCC TCCGATAGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AATCCTCTTG 60

CTGTTGCATC CGACTCGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCAG AGTGATTGAC 120

TACCCGTCTC GGGGGTCTTT CATTTGGGGG CTCGTCCGGG ATCTGGAGAC CCCTGCCCAG 180

GGACCACCGA CCCACCACCG GGAGGTAAGC TGGCCAGCAA TTGTTCTGTG TCTGTCCATT 240

GTCCTGTGTC TTTGATTGAT TTTATGCGCC TGTGTCTGTA CTAGTTGGCC GACTAGATTG 300

GTATCTGGCG GATCCGTGGT GGAACTGACG AGTTCGAGAC ACCCGGCCGC AACCCTGGGA 360

GACGTCCCAG GGACTTCGGG GGCCATTTTT GTGGCCCGGC CAGAGTCCAA CCATCCCGAT 420

CGTTTTGGAC TCTTTGGTGC ACCCCCCTTA GAGGAGGGGT ATGTGGTTCT GGTAGGAGAC 480

AGAGGGCTAA AACGGTTTCC GCCCCCGTCT GAGTTTTTGC TTTCGGTTTG GAACCGAAGC 540

CGCGCCGCGC GTCTTGTCTG CTGCAGCATC GTTCTGTGTT GTCTCTGTTT GACTGTTTTT 600

CTGTATTTGT CTGAAAACAT GGGCCAGGCT GTTACCACCC CCTTAAGTTT GACTTTAGAC 660

CACTGGAAGG ATGTCGAACG GACAGCCCAC AACCTGTCGG TAGAGGTTAG AAAAAGGCGC 720

TGGGTTACAT TCTGCTCTGC AGAATGGCCA ACCTTCAACG TCGGATGGCC ACGAGACGGC 780

ACTTTTAACC CAGACATTAT TACACAGGTT AAGATCAAGG TCTTCTCACC TGGCCCACAT 840

GGACATCCGG ATCAGGTCCC CTACATCGTG ACCTGGGAAG CTATAGCAGT AGACCCCCCT 900

CCCTGGGTCA GACCCTTCGT GCACCCTAAA CCTCCCCTCT CTCTTCCCCC TTCAGCCCCC 960

TCTCTCCCAC CTGAACCCCC ACTCTCGACC CCGCCCCAGT CCTCCCTCTA TCCGGCTCTC 1020

ACTTCTCCTT TAAACACCAA ACCTAGGCCT CAAGTCCTTC CTGATAGCGG AGGACCACTC 1080

ATTGATCTAC TCACGGAGGA CCCTCCGCCT TACCGGGACC CAGGGCCACC CTCTCCTGAC 1140

GGGAACGGCG ATAGCGGAGA AGTGGCCCCT ACAGAAGGAG CCCCTGACCC TTCCCCAATG 1200

GTATCCCGCC TGCGGGGAAG AAAAGAACCC CCCGTGGCGG ATTCTACTAC CTCTCAGGCG 1260

TTCCCCCTTC GCCTGGGAGG GAATGGACAG TATCAATACT GGCCATTTTC CTCCTCTGAC 1320

CTCTATAACT GGAAAAATAA CAACCCCTCT TTCTCCGAGG ACCCAGCTAA ATTGACAGCT 1380

TTGATCGAGT CCGTTCTCCT TACTCATCAG CCCACTTGGG ATGACTGCCA ACAGCTATTA 1440

GGGACCCTGC TGACGGGAGA AGAAAAACAG CGAGTGCTCC TAGAGGCCCG AAAGGCGGTT 1500

CGAGGGGAGG ACGGACGCCC AACTCAGCTG CCCAATGACA TTAATGATGC TTTTCCCTTG 1560

GAACGTCCCG ACTGGGACTA CAACACCCAA CGAGGTAGGA ACCACCTAGT CCACTATCGC 1620

CAGTTGCTCC TAGCGGGTCT CCAAAACGCG GGCAGAAGCC CCACCAATTT GGCCAAGGTA 1680

AAAGGGATAA CCCAGGGACC TAATGAGTCT CCCTCAGCCT TTTTAGAGAG ACTCAAGGAG 1740

GCCTATCGCA GATACACTCC TTATGACCCT GAGGACCCAG GGCAAGAAAC CAATGTGGCC 1800

ATGTCATTCA TCTGGCAGTC CGCCCCGGAT ATCGGGCGAA AGTTAGAGCG GTTAGAAGAT 1860

TTGAAGAGTA AGACCTTAGG AGACTTAGTG AGGGAAGCTG AAAAGATCTT TAATAAACGA 1920

GAAACCCCGG AAGAAAGAGA GGAACGTATT AGGAGAGAAA CAGAGGAAAA GGAAGAACGC 1980

CGTAGGGCAG AGGATGTGCA GAGAGAGAAG GAGAGGGACC GCAGAAGACA TAGAGAAATG 2040

AGTAAGTTGC TGGCTACTGT CGTTAGCGGG CAGAGACAGG ATAGACAGGG AGGAGAGCGA 2100

AGGAGGCCCC AACTCGACCA CGACCAGTGT GCCTACTGCA AAGAAAAGGG ACATTGGGCT 2160

AGAGATTGCC CCAAGAAGCC AAGAGGACCC CGGGGACCAC GACCCCAGGC CTCCCTCCTG 2220

ACCTTAGACG ATTAGGGAGG TCAGGGTCAG GAGCCCCCCC CTGAACCCAG GATAACCCTC 2280

AGAGTCGGGG GGCAACCCGT CACCTTCCTA GTGGATACTG GGGCCCAACA CTCCGTGCTG 2340

-continued

```
ACCCAAAATC CTGGACCCCT AAGTGACAAG TCTGCCTGGG TCCAAGGGGC TACTGGAGGG   2400

AAGCGGTATC GCTGGACCAC GGATCGCCGA GTGCACCTAG CCACCGGTAA GGTCACCCAT   2460

TCTTTCCTCC ATGTACCAGA TTGCCCCTAT CCTCTGCTAG GAAGAGATTT GCTGACTAAA   2520

CTAAAAGCCC AAATTCACTT TGAGGGATCA GGAGCTCAGG TTGTGGGACC AATGGGACAG   2580

CCCCTGCAAG TGCTGACCCT AAACATAGAA GATGAGTATC GGCTACATGA GACCTCAAAA   2640

GGGCCAGATG TGCCTCTAGG GTCCACATGG CTCTCTGATT TTCCCCAGGC CTGGGCAGAA   2700

ACCGGGGGCA TGGGGCTGGC CGTTCGCCAA GCTCCTCTGA TCATACCTCT GAAGGCAACC   2760

TCTACCCCCG TGTCCATAAA ACAATACCCC ATGTCACAAG AAGCCAGACT GGGGATCAAG   2820

CCCCACATAC AGAGACTGCT GGATCAGGGA ATTCTGGTAC CCTGCCAGTC CCCCTGGAAC   2880

ACGCCCCTGC TACCCGTTAA GAAACCGGGG ACTAATGATT ATAGGCCTGT CCAGGATCTG   2940

AGAGAAGTCA ACAAGCGGGT GGAAGACATC CACCCCACCG TGCCCAACCC TTACAACCTC   3000

TTGAGCGGGC TCCCACCGTC CCACCAGTGG TACACTGTGC TTGACTTAAA AGATGCTTTT   3060

TTCTGCCTGA GACTCCACCC CACCAGTCAG TCTCTCTTCG CCTTTGAGTG GAGAGATCCA   3120

GAGATGGGAA TCTCAGGACA ATTAACCTGG ACCAGACTCC CGCAGGGTTT CAAAAACAGT   3180

CCCACCCTGT TTGATGAAGC CCTGCACAGG GACCTCGCAG ACTTCCGGAT CCAGCACCCA   3240

GACCTGATTC TGCTCCAGTA TGTAGATGAC TTACTGCTGG CCGCCACTTC TGAGCTTGAC   3300

TGTCAACAAG GTACGCGGGC CCTGTTACAA ACCCTAGGGG ACCTCGGATA TCGGGCCTCG   3360

GCCAAGAAAG CCCAAATTTG CCAGAAACAG GTCAAGTATC TGGGGTATCT TCTAAAAGAG   3420

GGTCAGAGAT GGCTGACTGA GGCCAGAAAA GAGACTGTGA TGGGGCAGCC TACTCCGAAG   3480

ACCCCTCGAC AACTAAGGGA GTTCCTAGGG ACGGCAGGCT TCTGTCGCCT CTGGATCCCT   3540

GGGTTTGCAG AAATGGCAGC CCCCTTGTAC CCTCTCACCA AAACGGGGAC TCTGTTTGAG   3600

TGGGGCCCAG ACCAGCAAAA GGCCTACCAA GAGATCAAGC AGGCTCTCTT AACTGCCCCT   3660

GCCCTGGGAT TGCCAGACTT GACTAAGCCC TTCGAACTTT TTGTTGACGA GAAGCAGGGC   3720

TACGCCAAAG GTGTCCTAAC GCAAAAACTG GGGCCTTGGC GTCGGCCGGT GGCCTACCTG   3780

TCCAAAAAGC TAGACCCAGT GGCAGCTGGG TGGCCCCCTT GCCTACGGAT GGTAGCAGCC   3840

ATCGCCGTTC TGACCAAAGA CGCTGGCAAG CTCACCATGG GACAGCCACT AGTCATTCTG   3900

GCCCCCCATG CAGTAGAGGC ACTAGTTAAG CAACCCCCTG ATCGCTGGCT CTCCAACGCC   3960

CGAATGACCC ACTACCAGGC TCTGCTTCTG GACACGGACC GAGTCCAGTT CGGACCAATA   4020

GTGGCCCTAA ACCCAGCTAC GCTGCTCCCT CTACCTGAGG AGGGGCTGCA ACATGACTGC   4080

CTTGACATCT TGGCTGAAGC CCACGGAACT AGACCAGATC TTACGGACCA GCCTCTCCCA   4140

GACGCTGACC ACACCTGGTA CACAGATGGG AGCAGCTTCC TGCAAGAGGG GCAGCGCAAG   4200

GCCGGAGCAG CAGTAACCAC CGAGACCGAG GTAGTCTGGG CCAAAGCACT GCCAGCCGGG   4260

ACATCGGCCC AAAGAGCTGA GTTGATAGCG CTCACCCAAG CCTTAAAAAT GGCAGAAGGT   4320

AAGAAGCTGA ATGTTTACAC CGATAGCCGT TATGCTTTTG CCACTGCCCA TATTCACGGA   4380

GAAATATATA GAAGGCGCGG GTTGCTCACA TCAGAAGGAA AAGAAATCAA AAATAAGGAC   4440

GAGATCTTGG CCCTACTGAA GGCTCTCTTC CTGCCCAAAA GACTTAGCAT AATTCATTGC   4500

CCGGGACATC AGAAGGGAAA CCGCGCGGAG GCAAGGGGCA ACAGGATGGC CGACCAAGCG   4560

GCCCGAGAAG TAGCCACTAG AGAAACTCCA GAGACTTCCA CACTTCTGAT AGAAAATTCA   4620

GCCCCCTATA CTCATGAACA TTTTCACTAT ACGGTGACTG ACATAAAAGA TCTGACTAAA   4680

CTAGGGGCCA CTTATGACGA TGCAAAGAAG TGTTGGGTTT ATCAGGGAAA GCCTGTAATG   4740
```

-continued

```
CCTGATCAAT TCACCTTTGA ACTATTAGAT TTTCTTCATC AATTGACCCA CCTCAGTTTC   4800

TCAAAAACAA AGGCTCTTCT AGAAAGGAAC TACTGTCCTT ATTACATGCT GAACCGGGAT   4860

CGAACGCTCA AAGACATCAC TGAGACTTGC CAAGCCTGTG CACAGGTCAA TGCCAGCAAG   4920

TCTGCCGTCA AACAAGGGAC TAGAGTTCGA GGGCACCGAC CCGGCACCCA CTGGGAAATT   4980

GATTTCACTG AGGTAAAACC TGGCCTGTAT GGGTATAAAT ATCTTTTAGT TTTCATAGAC   5040

ACTTTCTCTG GATGGGTAGA AGCTTTCCCA ACCAAGAAAG AAACTGCCAA AGTTGTAACC   5100

AAGAAGCTAC TAGAAGAAAT CTTCCCCAGA TTCGGCATGC CACAGGTATT GGGAACCGAC   5160

AATGGGCCTG CCTTCGTCTC CAAGGTAAGT CAGACAGTAG CCGATTTACT GGGGGTTGAT   5220

TGGAAACTAC ATTGTGCTTA CAGACCCCAG AGTTCAGGTC AGGTAGAAAG AATGAATAGG   5280

ACAATCAAGG AGACTTTAAC TAAATTGACG CTTGCAACTG GCTCTAGGGA CTGGGTGCTC   5340

CTGCTTCCCC TAGCCCTGTA TCGAGCCCGC AACACGCCGG GCCCCCATGG TCTCACCCCA   5400

TATGAAATCT TATATGGGGC ACCCCCGCCC CTTGTAAACT TCCCTGATCC TGACATGGCA   5460

AAGGTTACTC ATAACCCCTC TCTCCAAGCC CATTTACAGG CACTCTACCT GGTCCAGCAC   5520

GAAGTCTGGA GACCGTTGGC GGCAGCTTAC CAAGAACAAC TGGACCGGCC GGTAGTGCCT   5580

CACCCTTTCC GAGTCGGTGA CACAGTGTGG GTCCGCAGAC ACCAAACTAA AAATCTAGAA   5640

CCCCGCTGGA AAGGACCTTA TACCGTCCTA CTGACTACCC CCACCGCTCT CAAAGTGGAC   5700

GGCATTGCAG CGTGGATCCA CGCTGCCCAC GTAAAGGCTG CCGACACCAG GATTGAGCCA   5760

CCATCGGAAT CGACATGGCG TGTTCAACGC TCTCAAAATC CCCTAAAGAT AAGATTGACC   5820

CGCGGGACCT CCTAATCCCC TTAATTCTCT TCCTGTCTCT CAAAGGGGCC AGATCCGCAG   5880

CACCCGGCTC CAGCCCTCAC CAGGTCTACA ACATTACCTG GGAAGTGACC AATGGGGATC   5940

GGGAGACAGT ATGGGCAATA TCAGGCAACC ACCCTCTGTG GACTTGGTGG CCAGTCCTCA   6000

CCCCAGATTT GTGTATGTTA GCTCTCAGTG GGCCGCCCCA CTGGGGGCTA GAGTATCAGG   6060

CCCCCTATTC CTCGCCCCCG GGGCCCCCTT GTTGCTCAGG GAGCAGCGGG AACGTTGCAG   6120

GCTGTGCCAG AGACTGCAAC GAGCCCTTGA CCTCCCTCAC CCCTCGGTGC AACACTGCCT   6180

GGAACAGACT TAAGCTGGAC CAGGTAACTC ATAAATCAAG TGAGGGATTT TATGTCTGCC   6240

CCGGGTCACA TCGCCCCCGG GAAGCCAAGT CCTGTGGGGG TCCAGACTCC TTCTACTGTG   6300

CCTCTTGGGG CTGCGAGACA ACCGGTAGAG TATACTGGAA GCCCTCCTCT TCTTGGGACT   6360

ACATCACAGT AGACAACAAT CTCACCTCTA ACCAGGCTGT TCAGGTATGC AAAGACAATA   6420

AGTGGTGCAA TCCCTTGGCT ATCCGGTTTA CAAACGCCGG GAAACAGGTC ACCTCATGGA   6480

CAACTGGACA CTATTGGGGT CTACGTCTTT ATGTCTCTGG ACAGGACCCA GGGCTTACTT   6540

TCGGGATCCG ACTCAGTTAT CAAAATCTAG GACCTCGGAT CCCAATAGGA CCAAACCCCG   6600

TCCTGGCAGA CCAACTTTCG TTCCCGCTAC CTAATCCCCT ACCCAAACCT GCCAAGTCTC   6660

CCCCCGCCTC TAGTTCGACT CCCACATTGA TTTCCCCGTC CCCCACTCCC ACTCAGCCCC   6720

CGCCAGCAGG AACGGGAGAC AGATTACTAA ATCTAGTACA GGGAGCTTAC CAGGCACTCA   6780

ACCTTACCAA CCCTGATAAA ACTCAAGAGT GCTGGTTATG CCTAGTGTCT GGACCCCCCT   6840

ATTACGAGGG GGTTGCCGTC CTAGGTACTT ATTCCAACCA TACCTCTGCC CCAGCTAACT   6900

GCTCCGTGGC CTCCCAACAC AAGCTGACCC TGTCCGAAGT GACTGGACGG GGACTCTGCA   6960

TAGGAACAGT CCCAAAAACT CACCAGGCCC TGTGCAACAC TACCCTTAAG GCAGGCAAAG   7020

GGTCTTACTA TCTAGTTGCC CCCACAGGAA CTATGTGGGC ATGTAACACT GGACTCACTC   7080

CATGCCTATC TGCCACCGTG CTTAATCGCA CCACTGACTA TTGCGTTCTC GTGGAATTAT   7140
```

-continued

GGCCCAGGGT CACCTACCAT CCTCCCAGTT ACGTCTATAG CCAGTTTGAA AAATCCCATA 7200

GACATAAAAG AGAACCAGTG TCCTTAACCT TGGCCTTATT ATTAGGTGGG CTAACTATGG 7260

GTGGCATCGC CGCGGGAGTA GGGACAGGAA CTACCGCCCT GGTCGCCACC CAGCAGTTTC 7320

AGCAGCTCCA TGCTGCCGTA CAAGATGATC TCAAAGAAGT CGAAAAGTCA ATTACTAACC 7380

TAGAAAAGTC TCTTACTTCG TTGTCTGAGG TTGTACTGCA GAATCGACGA GGCCTAGACC 7440

TGTTGTTCCT AAAAGAGGGA GGACTGTGTG CTGCCCTAAA AGAAGAATGT TGTTTCTATG 7500

CTGACCATAC AGGCCTAGTA AGAGATAGTA TGGCCAAATT AAGAGAGAGA CTCTCTCAGA 7560

GACAAAAACT ATTTGAGTCG AGCCAAGGAT GGTTCGAAGG ATGGTTTAAC AGATCCCCCT 7620

GGTTTACCAC GTTGATATCC ACCATCATGG GGCCTCTCAT TATACTCCTA CTAATTCTGC 7680

TTTTTGGACC CTGCATTCTT AATCGATTAG TTCAATTTGT TAAAGACAGG ATCTCAGTAG 7740

TCCAGGCTTT AGTCCTGACT CAACAATACC ACCAGCTAAA ACCACTAGAA TACGAGCCAC 7800

AATAAATAAA AGATTTTATT TAGTTTCCAG AAAAAGGGGG GAATGAAAGA CCCCACCAAA 7860

TTGCTTAGCC TGATAGCCGC AGTAACGCCA TTTTGCAAGG CATGGAAAAA TACCAAACCA 7920

AGAATAGAGA AGTTCAGATC AAGGGCGGGT ACACGAAAAC AGCTAACGTT GGGCCAAACA 7980

GGATATCTGC GGTGAGCAGT TTCGGCCCCG GCCCGGGGCC AAGAACAGAT GGTCACCGCG 8040

GTTCGGCCCC GGCCCGGGGC CAAGAACAGA TGGTCCCCAG ATATGGCCCA ACCCTCAGCA 8100

GTTTCTTAAG ACCCATCAGA TGTTTCCAGG CTCCCCCAAG GACCTGAAAT GACCCTGTGC 8160

CTTATTTGAA TTAACCAATC AGCCTGCTTC TCGCTTCTGT TCGCGCGCTT CTGCTTCCCG 8220

AGCTCTATAA AAGAGCTCAC AACCCCTCAC TCGGCGCGCC AGTCCTCCGA TAGACTGAGT 8280

CGCCCGGGTA CCCGTGTATC CAATAAATCC TCTTGCTGTT GCA 8323

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10367 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAACTCGAGC AGGGCTAGTA CAGACACAGG CGCATAAAAT CAATCAAAGA CACAGGACAA 60

TGGACAGACA CAGAACAATT GCTGGCCAGC TTACCTCCCG GTGGTGGGTC GGTGGTCCCT 120

GGGCAGGGGT CTCCAGATCC CGGACGAGCC CCCAAATGAA AGACCCCCGA GACGGGTAGT 180

CAATCACTCT GAGGAGACCC TCCCAAGGAA CAGCGAGACC ACGAGTCGGA TGCAACAGCA 240

AGAGGATTTA TTGGATACAC GGGTACCCGG GCGACTCAGT CTATCGGAGG ACTGGCGCGC 300

CGAGTGAGGG GTTGTGAGCT CTTTTATAGA GCTCGGGAAG CAGAAGCGCG CGAACAGAAG 360

CGAGAAGCAG GCTGATTGGT TAATTCAAAT AAGGCACAGG GTCATTTCAG GTCCTTGGGG 420

GAGCCTGGAA ACATCTGATG GGTCTTAAGA AACTGCTGAG GGTTGGGCCA TATCTGGGGA 480

CCATCTGTTC TTGGCCCCGG GCCGGGGCCG AACCGCGGTG ACCATCTGTT CTTGGCCCCG 540

GGCCGGGGCC GAAACTGCTC ACCGCAGATA TCCTGTTTGG CCCAACGTTA GCTGTTTTCG 600

TGTACCCGCC CTTGATCTGA ACTTCTCTAT TCTTGGTTTG GTATTTTTCC ATGCCTTGCA 660

AAATGGCGTT ACTGCGGCTA TCAGGCTAAG CAACTTGGTG GGGTCTTTCA TTCCCCCCTT 720

TTTCTGGAAA CTAAATAAAA TCTTTTATTT ATCATGGCTC GTATTCTAGT GGTTTTAGCT 780

GGTGGTATTG TTGAGTCAGG ACTAAAGCCT GGACTACTGA GATCCTGTCT TTAACAAATT 840

-continued

GAACTAATCG ATTCATTAGC TAGCTCCTGC TGGCGGGGGC TGAGTGGGAG TGGGGGACGG 900

GGAAATCAAT GTGGGAGTCG AATTAGAGGC GGGGGGAGAC TTGGCAGGTT TGGGTAGGGG 960

ATTAGGTCGC GGGAGCGAAA GTTGGTCTGC CAGGACGGGG TTCGGTCCTA TCGGGACCCG 1020

AGGTCCTAGA TTTTGATATC TGAGTCGGAT CCCGAAAGTA AGCCCCGGGT CCCGCCCAGA 1080

GACATAAAGA CGTAGACCCC AATAGTGTCC AGTTGTCCAT GAGGTGACCT GTTTCCCGGC 1140

GTTTGTAAAC TGGATAGCCA AGGGATTGCA CCACTTATTG TCTTTGCATA CCTGGACAGC 1200

CTGGCTAGTG GTGAGATTGT TGTCCACTGT GATGTAGTCC CAAGAGGAGG AGGGCTTCCA 1260

GTATACTCTA CCGGTTGTCT CGCAGCCCCA AGAGGCACAG TAGAAGGAGT CTGGACCTCC 1320

ACAGGACTTG GCTTCCCGGG GGCGATGTGA CCCGGGGCAG ACATAAAATC CCTCACTTGA 1380

TTTATGAGTT ACCTGGTCTA GCTTAAGTCT GTTCCAGGCA GTGTTGCACC GAGGGGTGAG 1440

GGAGGTCAAG GGCTCGTCGC AGTCTCTGGA ACAGCCTGCA CTGCTCCCGC TGCTCCCTGA 1500

GCAACAAGGG GGCCCCGGGG GCGAGGAATA GGGGGCCTGA TACTCTAGCC CCCAGTGGGG 1560

CGGCCCACTG AGAGCTAACA TACACAAATC TGGGGTGAGG ACTGGCCACC AAGTCCACAG 1620

AGGGTGGTTG CCTGATATTG CCCATACTGT CTCCCGATCC CCATTGGTCA CTTCCCAGGT 1680

AATGTTGTAG ACCTGGTGAG GGCTGGAGCC GGGTGCTGCG GATCTGGCCC CTTTGAGAGA 1740

CAGGAAGAGA ATTAAGGGGA TTAGGAGGTC CCGCGGGTCA ATCTTATCTT TAGGGGATTT 1800

TGGGAGCGTT GAACACGCCA TGTCGATTCT GCTGGTGGCT CAATCCTGGT GTCGGCAGCC 1860

TTTACGTGGG CAGCGTGGAT CCACGCTGCA ATGCCGTCTA CTTTGAGAGC GGTGGGGGTA 1920

GTCAGTAGGA CGGTATAGGG TCCTTTCCAG CGGGGTTCTA GATTTTTAGT TTGGTGTCTG 1980

CGGACCCACA CTGTGTCACC GACCCGGAAA GGGTGAGGTA CTACCGGCCG GTCTAGTTGC 2040

TCTTGGTAAG CTGCCGCCAA CGGTCTCCAG ACTTCGTGCT GGACCAGGTA GAGTGCCTGT 2100

AAATGAGCTT GGAGAGAGGG GTTATGAGTA ACCTTTGCCA TGTCAGGATC AGGGAAGTTT 2160

ACAAGGGGCG GGGGTGCCCC ATATAAGATT TCATATGGGG TGAGACCGTG GGGGCCCGGC 2220

GTGTTGCGGG CTCGATACAG GGCAAGGGGA AGCAGGAGCA CCCAGTCCCT AGAGCCAGTT 2280

GCAAGCGTCA ATTTAGTTAA AGTCTCCTTG ATTGTCCTAT TCATTCTTTC TACCTGACCT 2340

GAACTCTGGG GTCTGTAAGC ACAATGTAGT TTCCAATCAA CCCCCAATAA ATCGGCTACT 2400

GTCTGACTTA CCTTGGAGAC GAAGGCAGGC CCATTGTCGG TTCCCAATAC CTGTGGCATG 2460

CCGAATCTGG GGAAGATTTC TTCTAGTAGC TTCTTGGTTA CAACTTTGGC AGTTTCTTTC 2520

TTGGTTGGGA AAGCTTCTAC CCATCCAGAG AAAGTGTCTA TGAAAACTAA AAGATATTTA 2580

TACCCATACA GGCCAGGTTT TACCTCAGTG AAATCAATTT CCCAGTGGGT GCCGGGTCGG 2640

TGCCCTCGAA CTCTAGTCCC TTGTTTGACG GCAGACTTGC TGGCATTGAC CTGTGCACAG 2700

GCTTGGCAAG TCTCAGTGAT GTCTTTGAGC GTTCGATCCC GGTTCAGCAT GTAATAAGGA 2760

CAGTAGTTCC TTTCTAGAAG AGCCTTTGTT TTTGAGAAAC TGAGGTGGGT CAATTGATGA 2820

AGAAAATCTA ATAGTTCAAA GGTGAATTGA TCAGGCATTA CAGGCTTTCC CTGATAAACC 2880

CAACACTTCT TTGCATCGTC ATAAGTGGCC CCTAGTTTAG TCAGATCTTT TATGTCAGTC 2940

ACCGTATAGT GAAAATGTTC ATGAGTATAG GGGGCTGAAT TTTCTATCAG AAGTGTGGAA 3000

GTCTCTGGAG TTTCTCTAGT GGCTACTTCT CGGGCCGCTT GGTCGGCCAT CCTGTTGCCC 3060

CTTGCCTCCG CGCGGTTTCC CTTCTGATGT CCCGGGCAAT GAATTATGCT AAGTCTTTTG 3120

GGCAGGAAGA GAGCCTTCAG TAGGGCCAAG ATCTCGTCCT TATTTTTGAT TTCTTTTCCT 3180

TCTGATGTGA GCAACCCGCG CCTTCTATAT ATTTCTCCGT GAATATGGGC AGTGGCAAAA 3240

-continued

GCATAACGGC TATCGGTGTA AACATTCAGC TTCTTACCTT CTGCCATTTT TAAGGCTTGG 3300

GTGAGCGCTA TCAACTCAGC TCTTTGGGCC GATGTCCCGG CTGGCAGTGC TTTGGCCCAG 3360

ACTACCTCGG TCTCGGTGGT TACTGCTGCT CCGGCCTTGC GCTGCCCCTC TTGCAGGAAG 3420

CTGCTCCCAT CTGTGTACCA GGTGTGGTCA GCGTCTGGGA GAGGCTGGTC CGTAAGATCT 3480

GGTCTAGTTC CGTGGGCTTC AGCCAAGATG TCAAGGCAGT CATGTTGCAG CCCCTCCTCA 3540

GGTAGAGGGA GCAGCGTAGC TGGGTTTAGG GCCACTATTG GTCCGAACTG GACTCGGTCC 3600

GTGTCCAGAA GCAGAGCCTG GTAGTGGGTC ATTCGGGCGT TGGAGAGCCA GCGATCAGGG 3660

GGTTGCTTAA CTAGTGCCTC TACTGCATGG GGGGCCAGAA TGACTAGTGG CTGTCCCATG 3720

GTGAGCTTGC CAGCGTCTTT GGTCAGAACG GCGATGGCTG CTACCATCCG TAGGCAAGGG 3780

GGCCACCCAG CTGCCACTGG GTCTAGCTTT TTGGACAGGT AGGCCACCGG CCGACGCCAA 3840

GGCCCCAGTT TTTGCGTTAG GACACCTTTG GCGTAGCCCT GCTTCTCGTC AACAAAAAGT 3900

TCGAAGGGCT TAGTCAAGTC TGGCAATCCC AGGGCAGGGG CAGTTAAGAG AGCCTGCTTG 3960

ATCTCTTGGT AGGCCTTTTG CTGGTCTGGG CCCCACTCAA ACAGAGTCCC CGTTTTGGTG 4020

AGAGGGTACA AGGGGGCTGC CATTTCTGCA AACCCAGGGA TCCAGAGGCG ACAGAAGCCT 4080

GCCGTCCCTA GGAACTCCCT TAGTTGTCGA GGGGTCTTCG GAGTAGGCTG CCCCATCACA 4140

GTCTCTTTTC TGGCCTCAGT CAGCCATCTC TGACCCTCTT TTAGAAGATA CCCCAGATAC 4200

TTGACCTGTT TCTGGCAAAT TTGGGCTTTC TTGGCCGAGG CCCGATATCC GAGGTCCCCT 4260

AGGGTTTGTA ACAGGGCCCG CGTACCTTGT TGACAGTCAA GCTCAGAAGT GGCGGCCAGC 4320

AGTAAGTCAT CTACATACTG GAGCAGAATC AGGTCTGGGT GCTGGATCCG GAAGTCTGCG 4380

AGGTCCCTGT GCAGGGCTTC ATCAAACAGG GTGGGACTGT TTTTGAAACC CTGCGGGAGT 4440

CTGGTCCAGG TTAATTGTCC TGAGATTCCC ATCTCTGGAT CTCTCCACTC AAAGGCGAAG 4500

AGAGACTGAC TGGTGGGGTG GAGTCTCAGG CAGAAAAAAG CATCTTTTAA GTCAAGCACA 4560

GTGTACCACT GGTGGGACGG TGGGAGCCCG CTCAAGAGGT TGTAAGGGTT GGGCACGGTG 4620

GGGTGGATGT CTTCCACCCG CTTGTTGACT TCTCTCAGAT CCTGGACAGG CCTATAATCA 4680

TTAGTCCCCG GTTTCTTAAC GGGTAGCAGG GGCGTGTTCC AGGGGGACTG GCAGGGTACC 4740

AGAATTCCCT GATCCAGCAG TCTCTGTATG TGGGGCTTGA TCCCCAGTCT GGCTTCTTGT 4800

GACATGGGGT ATTGTTTTAT GGACACGGGG GTAGAGGTTG CCTTCAGAGG TATGATCAGA 4860

GGAGCTTGGC GAACGGCCAG CCCCATGCCC CCGGTTTCTG CCCAGGCCTG GGGAAAATCA 4920

GAGAGCCATG TGGACCCTAG AGGCACATCT GGCCCTTTTG AGGTCTCATG TAGCCGATAC 4980

TCATCTTCTA TGTTTAGGGT CAGCACTTGC AGGGGCTGTC CCATTGGTCC CACAACCTGA 5040

GCTCCTGATC CCTCAAAGTG AATTTGGGCT TTTAGTTTAG TCAGCAAATC TCTTCCTAGC 5100

AGAGGATAGG GGCAATCTGG TACATGGAGG AAAGAATGGG TGACCTTACC GGTGGCTAGG 5160

TGCACTCGGC GATCCGTGGT CCAGCGATAC CGCTTCCCTC CAGTAGCCCC TTGGACCCAG 5220

GCAGACTTGT CACTTAGGGG TCCAGGATTT TGGGTCAGCA CGGAGTGTTG GGCCCCAGTA 5280

TCCACTAGGA AGGTGACGGG TTGCCCCCCG ACTCTGAGGG TTATCCTGGG TTCAGGGGGG 5340

GGCTCCTGAC CCTGACCTCC CTAATCGTCT AAGGTCAGGA GGGAGGCCTG GGGTCGTGGT 5400

CCCCGGGGTC CTCTTGGCTT CTTGGGGCAA TCTCTAGCCC AATGTCCCTT TTCTTTGCAG 5460

TAGGCACACT GGTCGTGGTC GAGTTGGGGC CTCCTTCGCT CTCCTCCCTG TCTATCCTGT 5520

CTCTGCCCGC TAACGACAGT AGCCAGCAAC TTACTCATTT CTCTATGTCT TCTGCGGTCC 5580

CTCTCCTTCT CTCTCTGCAC ATCCTCTGCC CTACGGCGTT CTTCCTTTTC CTCTGTTTCT 5640

-continued

```
CTCCTAATAC GTTCCTCTCT TTCTTCCGGG GTTTCTCGTT TATTAAAGAT CTTTTCAGCT   5700

TCCCTCACTA AGTCTCCTAA GGTCTTACTC TTCAAATCTT CTAACCGCTC TAACTTTCGC   5760

CCGATATCCG GGGCGGACTG CCAGATGAAT GACATGGCCA CATTGGTTTC TTGCCCTGGG   5820

TCCTCAGGGT CATAAGGAGT GTATCTGCGA TAGGCCTCCT TGAGTCTCTC TAAAAAGGCT   5880

GAGGGAGACT CATTAGGTCC CTGGGTTATC CCTTTTACCT TGGCCAAATT GGTGGGGCTT   5940

CTGCCCGCGT TTTGGAGACC CGCTAGGAGC AACTGGCGAT AGTGGACTAG GTGGTTCCTA   6000

CCTCGTTGGG TGTTGTAGTC CCAGTCGGGA CGTTCCAAGG GAAAAGCATC ATTAATGTCA   6060

TTGGGCAGCT GAGTTGGGCG TCCGTCCTCC CCTCGAACCG CCTTTCGGGC CTCTAGGAGC   6120

ACTCGCTGTT TTTCTTCTCC CGTCAGCAGG GTCCCTAATA GCTGTTGGCA GTCATCCCAA   6180

GTGGGCTGAT GAGTAAGGAG AACGGACTCG ATCAAAGCTG TCAATTTAGC TGGGTCCTCG   6240

GAGAAAGAGG GGTTGTTATT TTTCCAGTTA TAGAGGTCAG AGGAGGAAAA TGGCCAGTAT   6300

TGATACTGTC CATTCCCTCC CAGGCGAAGG GGGAACGCCT GAGAGGTAGT AGAATCCGCC   6360

ACGGGGGGTT CTTTTCTTCC CCGCAGGCGG GATACCATTG GGGAAGGGTC AGGGGCTCCT   6420

TCTGTAGGGG CCACTTCTCC GCTATCGCCG TTCCCGTCAG GAGAGGGTGG CCCTGGGTCC   6480

CGGTAAGGCG GAGGGTCCTC CGTGAGTAGA TCAATGAGTG GTCCTCCGCT ATCAGGAAGG   6540

ACTTGAGGCC TAGGTTTGGT GTTTAAAGGA GAAGTGAGAG CCGGATAGAG GGAGGACTGG   6600

GGCGGGGTCG AGAGTGGGGG TTCAGGTGGG AGAGAGGGGG CTGAAGGGGG AAGAGAGAGG   6660

GGAGGTTTAG GGTGCACGAA GGGTCTGACC CAGGGAGGGG GGTCTACTGC TATAGCTTCC   6720

CAGGTCACGA TGTAGGGGAC CTGATCCGGA TGTCCATGTG GGCCAGGTGA GAAGACCTTG   6780

ATCTTAACCT GTGTAATAAT GTCTGGGTTA AAAGTGCCGT CTCGTGGCCA TCCGACGTTG   6840

AAGGTTGGCC ATTCTGCAGA GCAGAATGTA ACCCAGCGCC TTTTTCTAAC CTCTACCGAC   6900

AGGTTGTGGG CTGTCCGTTC GACATCCTTC CAGTGGTCTA AAGTCAAACT TAAGGGGGTG   6960

GTAACAGCCT GGCCCATGTT TTCAGACAAA TACAGAAAAA CAGTCAAACA GAGACAACAC   7020

AGAACGATGC TGCAGCAGAC AAGACGCGCG GCGCGGCTTC GGTTCCAAAC CGAAAGCAAA   7080

AACTCAGACG GGGGCGGAAA CCGTTTTAGC CCTCTGTCTC CTACCAGAAC CACATACCCC   7140

TCCTCTAAGG GGGGTGCACC AAAGAGTCCA AAACGATCGG GATGGTTGGA CTCTGGCCGG   7200

GCCACAAAAA TGGCCCCCGA AGTCCCTGGG ACGTCTCCCA GGGTTGCGGC CGGGTGTCTC   7260

GAACTCGTCA GTTCCACCAC GGATCCGCCA GATACCAATC TAGTCGGCCA ACTAGTACAG   7320

ACACAGGCGC ATAAAATCAA TCAAAGACAC AGGACAATGG ACAGACACAG AACAATTGCT   7380

GGCCAGCTTA CCTCCCGGTG GTGGGTCGGT GGTCCCTGGG CAGGGGTCTC CAGATCCCGG   7440

ACGAGCCCCC AAATGAAAGA CCCCCGAGAC GGGTAGTCAA TCACTCTGAG GAGACCCTCC   7500

CAAGGAACAG CGAGACCACG AGTCGGATGC AACAGCAAGA GGATTTATTG GATACACGGG   7560

TACCCGGGCG ACTCAGTCTA TCGGAGGACT GGCGCGCCGA GTGAGGGGTT GTGAGCTCTT   7620

TTATAGAGCT CGGGAAGCAG AAGCGCGCGA ACAGAAGCGA GAAGCAGGCT GATTGGTTAA   7680

TTCAAATAAG GCACAGGGTC ATTTCAGGTC CTTGGGGGAG CCTGGAAACA TCTGATGGGT   7740

CTTAAGAAAC TGCTGAGGGT TGGGCCATAT CTGGGGACCA TCTGTTCTTG GCCCCGGGCC   7800

GGGGCCGAAC CGCGGTGACC ATCTGTTCTT GGCCCCGGGC CGGGGCCGAA ACTGCTCACC   7860

GCAGATATCC TGTTTGGCCC AACGTTAGCT GTTTTCGTGT ACCCGCCCTT GATCTGAACT   7920

TCTCTATTCT TGGTTTGGTA TTTTTCCATG CCTTGCAAAA TGGCGTTACT GCGGCTATCA   7980

GGCTAAATCA GATCTGCCGG TCTCCCTATA GTGAGTCGTA TTAATTTCGA TAAGCCAGGT   8040
```

-continued

```
TAACCTGCAT TAATGAATCG GCCAACGCGC GGGGAGAGGC GGTTTGCGTA TTGGGCGCTC   8100

TTCCGCTTCC TCGCTCACTG ACTCGCTGCG CTCGGTCGTT CGGCTGCGGC GAGCGGTATC   8160

AGCTCACTCA AAGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG CAGGAAAGAA   8220

CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT   8280

TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG   8340

GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG   8400

CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG   8460

CGTGGCGCTT TCTCAATGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC   8520

CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA   8580

CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG   8640

TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA AGTGGTGGCC   8700

TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC   8760

CTTCGGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG   8820

TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT   8880

GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT   8940

CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA   9000

ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT TAATCAGTGA   9060

GGCACCTATC TCAGCGATCT GTCTATTTCG TTCATCCATA GTTGCCTGAC TCCCCGTCGT   9120

GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG   9180

AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA   9240

GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCGGGA   9300

AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTTGCCA TTGCTACAGG   9360

CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC   9420

AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC   9480

GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA   9540

TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC   9600

CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG CGTCAATACG   9660

GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA AACGTTCTTC   9720

GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG   9780

TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC   9840

AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT GAATACTCAT   9900

ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA TGAGCGGATA   9960

CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT TTCCCCGAAA  10020

AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG  10080

TATCACGAGG CCCTTTCGTC TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT  10140

GCAGCTCCCG GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG  10200

TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA  10260

GCAGATTGTA CTGAGAGTGC ACCATATGGA CATATTGTCG TTAGAACGCG GCTACAATTA  10320

ATACATAACC TTATGTATCA TACACATACG ATTTAGGTGA CACTATA                 10367
```

-continued

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Ala Cys Ser Thr Leu Pro Lys Ser Pro Lys Asp Lys Ile Asp Pro
  1               5                  10                  15

Arg Asp Leu Leu Ile Pro Leu Ile Leu Phe Leu Ser Leu Lys Gly Ala
             20                  25                  30

Arg Ser Ala Ala Pro Gly Ser Ser Pro His Gln Val Tyr Asn Ile Thr
         35                  40                  45

Trp Glu Val Thr Asn Gly Asp Arg Glu Thr Val Trp Ala Ile Ser Gly
     50                  55                  60

Asn His Pro Leu Trp Thr Trp Trp Pro Val Leu Thr Pro Asp Leu Cys
 65                  70                  75                  80

Met Leu Ala Leu Ser Gly Pro Pro His Trp Gly Leu Glu Tyr Gln Ala
                 85                  90                  95

Pro Tyr Ser Ser Pro Pro Gly Pro Pro Cys Cys Ser Gly Ser Ser Gly
            100                 105                 110

Ser Ser Ala Gly Cys Ser Arg Asp Cys Asp Glu Pro Leu Thr Ser Leu
        115                 120                 125

Thr Pro Arg Cys Asn Thr Ala Trp Asn Arg Leu Lys Leu Asp Gln Val
    130                 135                 140

Thr His Lys Ser Ser Glu Gly Phe Tyr Val Cys Pro Gly Ser His Arg
145                 150                 155                 160

Pro Arg Glu Ala Lys Ser Cys Gly Gly Pro Asp Ser Phe Tyr Cys Ala
                165                 170                 175

Ser Trp Gly Cys Glu Thr Thr Gly Arg Val Tyr Trp Lys Pro Ser Ser
            180                 185                 190

Ser Trp Asp Tyr Ile Thr Val Asp Asn Asn Leu Thr Thr Ser Gln Ala
        195                 200                 205

Val Gln Val Cys Lys Asp Asn Lys Trp Cys Asn Pro Leu Ala Ile Gln
    210                 215                 220

Phe Thr Asn Ala Gly Lys Gln Val Thr Ser Trp Thr Thr Gly His Tyr
225                 230                 235                 240

Trp Gly Leu Arg Leu Tyr Val Ser Gly Arg Asp Pro Gly Leu Thr Phe
                245                 250                 255

Gly Ile Arg Leu Arg Tyr Gln Asn Leu Gly Pro Arg Val Pro Ile Gly
            260                 265                 270

Pro Asn Pro Val Leu Ala Asp Gln Leu Ser Leu Pro Arg Pro Asn Pro
        275                 280                 285

Leu Pro Lys Pro Ala Lys Ser Pro Pro Ala Ser Asn Ser Thr Pro Thr
    290                 295                 300

Leu Ile Ser Pro Ser Pro Thr Pro Thr Gln Pro Pro Pro Ala Gly Ala
305                 310                 315                 320

Ser Glx Glx
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 289 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Ala Ala Pro Gly Ser Ser Pro His Gln Val Tyr Asn Ile Thr Trp Glu
  1               5                  10                  15

Val Thr Asn Gly Asp Arg Glu Thr Val Trp Ala Ile Ser Gly Asn His
             20                  25                  30

Pro Leu Trp Thr Trp Trp Pro Val Leu Thr Pro Asp Leu Cys Met Leu
         35                  40                  45

Ala Leu Ser Gly Pro Pro His Trp Gly Leu Glu Tyr Gln Ala Pro Tyr
     50                  55                  60

Ser Ser Pro Pro Gly Pro Pro Cys Cys Ser Gly Ser Ser Gly Ser Ser
 65                  70                  75                  80

Ala Gly Cys Ser Arg Asp Cys Asp Glu Pro Leu Thr Ser Leu Thr Pro
                 85                  90                  95

Arg Cys Asn Thr Ala Trp Asn Arg Leu Lys Leu Asp Gln Val Thr His
            100                 105                 110

Lys Ser Ser Glu Gly Phe Tyr Val Cys Pro Gly Ser His Arg Pro Arg
        115                 120                 125

Glu Ala Lys Ser Cys Gly Gly Pro Asp Ser Phe Tyr Cys Ala Ser Trp
    130                 135                 140

Gly Cys Glu Thr Thr Gly Arg Val Tyr Trp Lys Pro Ser Ser Ser Trp
145                 150                 155                 160

Asp Tyr Ile Thr Val Asp Asn Asn Leu Thr Thr Ser Gln Ala Val Gln
                165                 170                 175

Val Cys Lys Asp Asn Lys Trp Cys Asn Pro Leu Ala Ile Gln Phe Thr
            180                 185                 190

Asn Ala Gly Lys Gln Val Thr Ser Trp Thr Thr Gly His Tyr Trp Gly
        195                 200                 205

Leu Arg Leu Tyr Val Ser Gly Arg Asp Pro Gly Leu Thr Phe Gly Ile
    210                 215                 220

Arg Leu Arg Tyr Gln Asn Leu Gly Pro Arg Val Pro Ile Gly Pro Asn
225                 230                 235                 240

Pro Val Leu Ala Asp Gln Leu Ser Leu Pro Arg Pro Asn Pro Leu Pro
                245                 250                 255

Lys Pro Ala Lys Ser Pro Pro Ala Ser Asn Ser Thr Pro Thr Leu Ile
            260                 265                 270

Ser Pro Ser Pro Thr Pro Thr Gln Pro Pro Pro Ala Gly Ala Ser Glx
        275                 280                 285

Glx
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
TCCTGGCGCC TCTAATTCGA CTCCCACATT                                    30
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTGCGGCGCC ACGGGAGACA GGTTACTAAA TC 32

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACTGATCGAT TCATTAGGCG CCGGATACCT TTGGACAGGC C 41

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CATCGCTAGC GTAACGCACA GTTTTAATTG TGGA 34

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTGATCGAT CTATTAGGCG CCCCCTGTAA TATTTGAACA T 41

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACTGTCTAGA AAGCGCGCGA ACAGAAGCGA GAAGC 35

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Cys Trp Leu Cys
1

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) FEATURE:
(B) LOCATION: 2...7
(D) OTHER INFORMATION: where Xaa at positions 1-7 is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Cys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ile Glu Gly Arg
1

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) FEATURE:
(B) LOCATION: 3...3
(D) OTHER INFORMATION: where Xaa at position 3 is a heterologous sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala Ser Xaa Gly Ala
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) FEATURE:
(B) LOCATION: 1...16
(D) OTHER INFORMATION: /note= “An analogous peptide matching the consensus sequence for HXB2d V2 domain and homologs with an additional C-terminal (Cys) as defined in the Los Alamos Human Retrovirus and AIDS database (ADP 794.1).”

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

-continued

Ile Arg Asp Lys Val Gln Lys Glu Tyr Ala Leu Phe Tyr Lys Leu Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Thr Arg Pro Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg Gly Pro
1 5 10 15

Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asn Met Arg Gln Ala
20 25 30

His

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Lys
1 5 10 15

Asn

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Val Pro Arg Gly Ser
1 5

We claim:

1. A fusion glycoprotein expressed by a vector, said fusion glycoprotein comprising (a) an amino acid sequence that has biological activity and is from a protein that lacks a hydrophobic glycosylation signal located about seven residues N-terminal to a Cys-Trp-Leu-Cys sequence (SEQ ID NO:18), said amino acid sequence being operably linked to the C-terminus of (b) a polypeptide sequence from a retroviral env surface protein, which polypeptide sequence comprises an N-terminal fragment of said retroviral env surface protein, wherein said fragment includes all the Cys residues of the N-terminal globular domain of said retroviral env surface protein, and wherein said retroviral env surface protein contains an N-glycan attachment site within a hydrophobic glycosylation signal located about seven residues N-terminal to a Cys-Trp-Leu-Cys sequence (SEQ ID NO:18).

2. The fusion protein of claim **1**, wherein said polypeptide sequence comprises at least a portion of an interdomain linker region extending from the last Cys residue of said N-terminal globular domain to said N-glycan attachment site.

3. The fusion protein of claim **2**, wherein said polypeptide sequence further comprises the C-terminal globular domain of said env surface protein operably linked to the C-terminus of said amino acid sequence.

4. The fusion glycoprotein of claim **3**, wherein said polypeptide sequence comprises said retroviral env surface protein.

5. The fusion glycoprotein of claim **3**, wherein said amino acid sequence is operably inserted within said at least a portion of the interdomain linker.

6. The fusion glycoprotein of claim **1**, wherein said vector comprises a gene encoding a protein that functions as a retroviral env gene for virus infection.

7. The fusion glycoprotein of claim **1**, wherein said amino acid sequence is less than 150 amino acids long.

8. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the V2 region of gp120 of HIV-1 or a glycosylated fragment thereof.

9. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the V1 region of gp120 of HIV-1 or a glycosylated fragment thereof.

10. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the V3 region of gp120 of HIV-1 or a glycosylated fragment thereof.

11. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the amino acid sequence of the V1/V2 domain of gp120 of HIV-1.

12. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the V3 domain of gp120 of HIV-1.

13. The fusion glycoprotein of claim 1, wherein said amino acid sequence comprises the V2 domain of gp120 of HIV-1.

14. The fusion glycoprotein of claim 1, wherein said amino acid sequence is amino acid sequence 86–179 of gp120 of the HXB2d strain, or a corresponding sequence of another HIV-1 strain.

15. The fusion glycoprotein of claim 1, wherein said amino acid sequence is amino acid sequence 261–306 of gp120 of the HXB2d strain, or a corresponding sequence of another HIV-1 strain.

16. The fusion glycoprotein of claim 1, wherein said vector is a retroviral particle.

17. The fusion glycoprotein of claim 16, wherein said retroviral particle is a Murine Leukemia Virus (MuLV) particle.

18. A retroviral particle comprising a recombinant gene encoding the fusion glycoprotein of claim 1.

19. The retroviral particle of claim 18, wherein said retroviral particle is a MuLV particle.

* * * * *